(51) International Patent Classification 5 : G01N 1/00, 33/48, A61K 37/66 A61K 45/05	**A1**	(11) International Publication Number: **WO 91/04479** (43) International Publication Date: 4 April 1991 (04.04.91)

(21) International Application Number: PCT/US90/05457

(22) International Filing Date: 25 September 1990 (25.09.90)

(30) Priority data:
412,294	25 September 1989 (25.09.89)	US
422,791	18 October 1989 (18.10.89)	US

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(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent)*, DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: EVALUATIVE MEANS FOR DETECTING INFLAMMATORY REACTIVITY AND FOR PREDICTING RESPONSE TO STRESS

(57) Abstract

The present invention relates to a diagnostic test for testing susceptibility of individuals to inflammatory diseases such as rheumatoid arthritis and to means to evaluate potential treatment of arthritis with therapeutic agents directed at the central nervous system (CNS), designed to by-pass the CNS defect. The invention also provides means for evaluating and predicting response to stress in individuals. A counter-regulatory feedback loop exists between the immune and central nervous systems, in which the immune or pro-inflammatory mediators stimulate corticotropin-releasing hormone (CRH) activation of the hypothalamic-pituitary-adrenal (HPA) axis hormonal cascade. The resultant increase in plasma glucocorticoids serves to restrain and limit the intensity of the inflammatory-immune response, through the potent immunosuppressive/anti-inflammatory actions of the glucocorticoids.

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EVALUATIVE MEANS FOR DETECTING INFLAMMATORY REACTIVITY AND FOR PREDICTING RESPONSE TO STRESS

BACKGROUND OF THE INVENTION

5   The present invention relates to a diagnostic test for testing susceptibility of individuals to inflammatory diseases such as rheumatoid arthritis and to means to evaluate potential treatment of arthritis with therapeutic agents directed at the central nervous system (CNS), designed to by-pass the CNS defect. The invention also
10   provides means for evaluating and predicting response to stress in individuals.

       A counter-regulatory feedback loop exists between the immune and central nervous systems, in which the immune or pro-inflammatory mediators stimulate cort-
15   icotropin-releasing hormone (CRH) activation of the hypothalamic-pituitary-adrenal (HPA) axis hormonal cascade. The resultant increase in plasma glucocorticoids serves to restrain and limit the intensity of the inflammatory-immune response, through the potent
20   immunosuppressive/anti-inflammatory actions of the glucocorticoids.

SUMMARY OF THE INVENTION

       It is an object of the present invention to provide a method for testing mammals for susceptibility to
25   inflammatory diseases. In its broadest aspect, the method comprises the steps of administering to a mammal a compound which is effective in stimulating the hypothalamic-pituitary-adrenal (HPA) axis and measuring the level of hormones secreted by the pituitary and adrenal glands of
30   the mammal. In a more specific aspect, the method comprises the steps of administering to a mammal a compound selected from the group consisting of cytokines, cell growth factors, neuroendocrine hormones such as corticotropin releasing hormone (CRH) or arginine vasopressin
35   (AVP), biogenic amines, agonists of biogenic amines, antagonists of biogenic amines, analogues of biogenic amines, monoamine oxidase inhibitors and biogenic amine uptake inhibitors or glucocorticoid receptor antagonists

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and measuring the level of glucocorticoids or adrenocorti-
cotropic (ACTH) in the blood plasma of the mammal. The
substance which is administered should not be the same as
the material which is measured.

5   The invention is useful as a model in the study of
the mammalian autoimmune diseases. Laboratory animals
which may serve as a good model in studying human systems
include rats, mice, guinea pigs, rabbits and chickens.
However, an ultimate objective of this invention is to
10   provide a method for diagnosing the susceptibility of
humans to inflammatory diseases.

          The invention is also useful as a means of pre-
dicting intensity of response to stress and capacity to
mount a sustained adaptive response to stress. The use of
15   therapy through CRH activation during an injury or illness
can serve the coherently related goals of preventing the
immune/inflammatory response from overshooting and of
restraining exploratory behavior to diminish exposure to
further danger.

20   The hormones to be measured should be hormones
which are secreted in increased levels by normal
individuals when the compound is administered to the
individual but which are not secreted in such high levels
after administration of the compound in individuals having
25   an inflammatory disease or susceptibility to an inflam-
matory disease. Hormones secreted by the pituitary and
adrenal glands which can be measured include glucocorti-
coids such as corticosterone, cortisol, and ACTH. Other
hormones which can be measured include CRH, prolactin,
30   arginine vasopressin (AVP), growth hormone (GH), thyroid
stimulating hormone (TSH), and endorphins/enkephalins.

          The compound which is used in the test is prefer-
ably administered intravenously (i.v.), however, other
modes of administration such as subcutaneously (s.c.) or
35   orally (p.o.) may be used. The compound is administered
together with a suitable non-toxic pharmaceutically
acceptable carrier in an amount sufficient to stimulate
the hypothalamic-pituitary-adrenal axis. The compound

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should be administered at a time when the hypothalamic-pituitary-adrenal axis is quiescent, i.e., in humans at 8 p.m. However, it could be administered between 8 a.m. - 10 a.m., for example, when giving compounds such as AVP. When an immune/inflammatory mediator such as interleukin-1 is used, the immune inflammatory mediator would probably be administered in a dose of 0.1 μg/kg to 10 μg/kg of body weight, preferably 1 μg/kg to 5 μg/kg of body weight. When CRH is used, 1 μg ovine CRH per kg body weight is administered i.v. When AVP is used, 0.01 to 2.0 mIU/kg/min is infused i.v. When a biogenic amine or analogue thereof such as quipazine is used, the compound would probably be administered in a dose of 0.01 to 1mg of quipazine per kg of body weight, preferably 0.1 to 0.5 mg/kg of body weight. Doses for other biogenic amines or analogues thereof should be determined on a case-by-case basis.

After administration of the compound it is necessary to wait for a time sufficient to allow the compound to raise the glucocorticoid or ACTH level in the blood plasma of the patient before testing. Generally, it is necessary to wait at least 10 minutes before testing. The glucocorticoid or ACTH level should be measured before the level returns to normal. The glucocorticoid or ACTH level may return to normal within 4 hours after administration of the compound. A preferred waiting period is 15 minutes to 2 hours after administration, more preferably 30 to 60 minutes after administration. If the hormone levels are significantly lower than (such as more than two standard deviations below) the mean established in normal individuals, individuals, then the patient has tested positive for possible susceptibility to inflammatory diseases.

The method is potentially useful for testing for inflammatory diseases including, but not limited to, arthritis, uveoretinitis, pneumonitis, encephalomyelitis, myocarditis, thyroiditis, nephritis, sialoadenitis, adrenalitis, orchitis, multiple sclerosis and hepatic granulomatous diseases.

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Various immune/inflammatory mediators may be used. Cytokines such as any one of the interleukins (interleukin-1 (IL-1), interleukin-2 (IL-2), interleukin-3 (IL-3), interleukin-4 (IL-4), interleukin-5 (IL-5) and interleukin-6 (IL-6)), interferons (alpha interferon, beta interferon and gamma interferon) or tumor necrosis factor (TNF) may be used in the test. Other cytokines such as epidermal growth factor (EGF), transforming growth factor-alpha and/or beta (TGF-alpha and/or TGF-beta) may also be used. Biogenic amines such as serotonin, norepinephrine, epinephrine, or dopamine may be used. In addition, analogs and agonists of these biogenic amines such as quipazine may also be used. Additional compounds which may be used include monamine oxidase inhibitors such as tranylcypromine sulfate (30 mg/patient) or isocarboxazid (30 mg/patient) which increase endogenous levels of biogenic amines. Biogenic amine uptake inhibitors such as fluoxetine may also be used.

It is known that inflammatory mediators such as IL-1 cause an increase in plasma corticosterone and ACTH possibly by stimulating the hypothalamic-pituitary-adrenal (HPA) axis. The present invention is based on the finding that susceptibility to arthritis and other inflammatory diseases is related to lack of HPA axis responsiveness to inflammatory mediators and other compounds.

The present invention is also potentially useful as a guide for the treatment of arthritis with agents that may bypass the HPA defect by stimulating the HPA axis centrally or at multiple levels. Such drugs would include the drugs listed below:

Neurotransmitters/monoamines/neuroexcitatory agents:

    serotonin agonists/releasers/uptake inhibitors:

        quipazine

        1-metachloro-phenyl-piperazine (mCPP)

        fenfluoramine

        fluoxetine

    adrenergic agonists/antagonists/uptake inhibitors:

          idasoxan

          yohimbine

          methoxamine

          desmethylimipramine

5   ritalin

     cholinergic agents:

          arecholine

          nicotine

     GABA agonists/antagonists:

10   BCCM-beta carboline

          FG 7142 (Sandoz)

          RO 15788

     MAO inhibitors

          MAO A:chlorgyline

15   MAO B:phenylzine

          isocarboxazid

          tranylcypromine

     Dopamine uptake inhibitors/releasers

          buproprion

20   amphetamine

     Excitatory amino acids/neuroexcitatory agents:

          glutamate

          cocaine

   Neurohormones:

25   rat/human corticotropin releasing hormone (CRH)

          corticotropin (ACTH)

          dexamethasone

          arginine vasopressin (AVP)

          thyroxin

30   thyroid stimulating hormone (TSH)

          estrogen

          progesterone

          testosterone

          Diapid (Bissendorff, LHRH antagonist)

35   It may also provide a guide for determination of dosage and timing schedule of replacement steroids or other HPA axis hormones such as CRH or ACTH.

     The above noted agents have been selected for

possible treatment of inflammatory diseases such as rheumatoid arthritis because they represent a variety of classes of neuroactive agents which would be expected to activate the CRH and/or related arousal systems on a long-term basis (i.e., without inducing tolerance). Such an effect would correct the putative pathophysiological defect in rheumatoid arthritis and, hence, significantly ameliorate inflammatory and/or affective symptoms associated with this illness. Of the above-mentioned compounds, it is expected that 1-metachloro-phenyl-piperazine (mCPP) (an antidepressant), fluoxetine (an antidepressant), idasoxan (an antidepressant), nicotine, FG 7142 (Sandoz), MAO A:chlorgyline (an antidepressant), and MAO B:phenylzine (an antidepressant) are expected to be preferred compounds for further study. These compounds will probably be administered in the same manner as recommended for their already known indications such antidepressants. Specifically, these compounds should be administered in an amount effective to stimulate the HPA axis which would bypass the defect in the HPA axis. It is also expected that analogues and/or derivatives of the above compounds may be useful.

As indicated in the examples below wherein controlled stress was introduced into the environment, activity may be used as a means of evaluating the level of hormones secreted by the pituitary or adrenal glands of the mammal being tested.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B show the severity of arthritis post-streptococcal cell wall (SCW) injection in euthymic versus athymic LEW and F344 rats. Severity of arthritis was quantitated by articular index (maximum of 16) for up to 42 days following a sign p. SCW injection. Data represent the mean ± S.E.M. for 5 rats per experimental group.

Figures 2A through 2D show the plasma corticosterone levels induced by SCW, IL-1 alpha, or quipazine in inbred F344/N and LEW/N rats, and in outbred HSD rats. Rats of each strain were injected i.p. with one mediator,

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as shown: SCW, (2 mg cell wall rhamnose), 1 μgm
recombinant IL-1 alpha, 1 mg quipazine or PBS control.
Corticosterone was determined in plasma collected 60
minutes post-injection. Horizontal lines represent means

5   of each group.

Figures 3A through 3F show the time course of
plasma ACTH and corticosterone responses to SCW, (panels A
and B); human recombinant IL-1 alpha (IL-1) (panels C and
D); or quipazine (QUIP) (panels E and F) in F344/N (-ᵃ-)

10   Versus LEW/N (-o-) rats. Plasma ACTH and corticosterone
were quantitated by radioimmunoassay at various time
points up to 4 hours following i.p. injection of each
agent shown. Data shown are mean ± S.E.M. of a minimum of
5 animals per experimental group.

15   Figures 4A through 4F show the dose responses of
plasma ACTH and corticosterone responses to SCW (panels A
and B); human recombinant IL-1 alpha (IL-1) (panels C and
D); or quipazine (QUIP) (panels E and F) in F344/N (-ᵃ-)
versus LEW/N (-o-) rate. Various doses of mediators shown

20   were injected i.p. and plasma ACTH and corticosterone were
quantitated by radioimmunoassay 60 minutes post-injection.
Data shown are mean ± S.E.M. of a minimum of 5 animals per
group.

Figures 5A and 5B show the dose responses of

25   plasma ACTH and corticosterone to various concentrations
of human CRH. CRH was injected i.p., and plasma ACTH and
corticosterone were measured by radioimmunoassay 60
minutes post injection. Data are mean ± S.E.M. of a
minimum of 5 animals per experimental group.

30   Figures 6A through 6D show CRH (A) and enkephalin
(B) transcript levels in the parvocellular neurons of the
paraventricular nucleus (PVN) were increased by SCW
administration in F344/N rats (ᵃ) but not in LEW/N
(o)rats. CRH (C) and enkephalin (D) transcript levels in

35   the PVN were not increased by rIL-1 alpha administration
in F344/N (ᵃ) or LEW/N (o) rats. F344/N and LEW/N rats
were injected intraperitoneally with 2 mg cell wall
rhamnose/100gm rat, or rIL-1 alpha, 1 μgm/100gm rat, and

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were sacrificed 0, 2, 4, and 7 hours later. In situ
hybridization and determination of the number of copies of
probe hybridized per section per PVN were performed as
previously described (6, 7). CRH mRNA levels were clearly

5   increased at 4 and 7 hours post-SCW injection in the
F344/N rats, both in comparison with baseline and LEW/N
levels (e.g., two-tailed T test, p<0.001 at 7 hours
between strains). Although enkephalin mRNA levels rose
above baseline in both strains in response to SCW adminis-

10   tration, the F344/N response was greater than the LEW/N
response (p.<0.05). A minimum of 6 rats were used for
each experimental condition, except the 2 hour time point,
for which 4 rats were used. Bars represent S.E.M.

        Figures 7A and 7B show the total hypothalamic

15   immuno-reactive CRH (iCRH) content in F344/N (A) and LEW/N
(B) rats, measured 4 hours after intraperitoneal injection
of various agents. F344/N (A) or LEW/N (B) rats were
either untreated or were injected intraperitoneally with
PBS, rIL-1 alpha, (1 μgm per rat), or SCW (2 mg cell wall

20   rhamnose per rat), and iCRH hypothalamic content was
quantitated by radioimmunoassay (10). A minimum of 10
rats per experimental condition was used. Statistical
significance was determined by one-way ANOVA followed by
Duncan's multiple range test. There was no significant

25   difference between untreated and PBS-treated F344/N rats,
nor between any treatment group of LEW/N rats by either
criterion. Both rIL-1 alpha and SCW-treated F344/N
hypothalami contained significantly more CRH than PBS or
untreated F344/N hypothalami, than treated or untreated

30   LEW/N hypothalami (* = ANOVA p<0.0001, Duncan's multiple
range p<0.05). Baseline levels of CRH in LEW/N hypothala-
mi were not significantly different from baseline CRH
levels in F344/N hypothalami by Duncan's multiple range
test.

35   Figure 8 shows the hypothalamic iCRH secretion
from F344/N (ᵃ) versus LEW/N (ᵒ) rats stimulated in vitro
with recombinant IL-1 alpha. Hypothalami from LEW/N or
F344/N rats were stimulated for 20 minute periods in vitro

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with control medium or with IL-1 alpha, at concentrations ranging from 10^{-13}M to 10^{-6}M. iCRH in the culture medium was quantitated by radioimmunoassay (11). Hypothalami from F344/N rats showed an increase of 150% over baseline
5   iCRH secretion, compared to a 10% increase over baseline by LEW/N hypothalami (* = p<0.0001). Statistical significance was determined by Duncan's multiple range test. A minimum of 7 rats per experimental condition was used.

                    Figures   9A   through   9F   show   the   plasma
10   corticosterone (9a) and ACTH (9b) levels in F344/N rats and LEW/N rats exposed to a variety of behavioral stresses versus controls. Rats were exposed to a variety of stressor prior to decapitation for collection of blood. Plasma ACTH and corticosterone were measured by radioimmu-
15   noassay (R.I.A.). Control = rats maintained in a stress free environment while immediately prior to decapitation for collection of blood; stress control = rats housed in the same room as the stressed rats during the period of stress, but not during decapitation; open field = rats
20   exposed   to   the   novel   environment   of   an   open   field; restraint = rats restrained for 1 hour; swim = animals exposed to swim stress; ether = rats which were exposed to 10,00- ppm ether for 1 hour. Data represent values obtain from two separate experiments. Horizontal lines represent
25   means ± S.E.M.; represents mean ACTH or corticosterone levels which were significantly different from controls (p<0.05 by the Duncan multiple range test); represents mean F344/N and LEW/N plasma ACTH or corticosterone levels which differ significantly from each other in response to
30   the   same   stress   (p<0.05   by   the   Duncan   multiple   range test.)

                    Figure   10   shows   the   CRH   mRNA   levels   in   the paraventricular   nucleus   of   the   hypothalamus   during restraint stress in F344/N versus LEW/N rats. Rats were
35   restrained for 1,1 or 3 hours prior to decapitation for collection of brains for _in situ_ hybridization for CRH mRNA of the hypothalamus. _In situ_ hybridization was preformed as described by Young (_Methods in Enzymology,_

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Vol. 168. Hormone Action. Park K. Neuroendocrine
Peptides, (Academic Press, New York, 1989) pp. 702-710.)
Briefly, brains were removed and frozen by immersion in
isopentane on dry ice. Sections were cut through the PVN
of the hypothalamus or through the anterior pituitary
using a cryostat and thaw-mounted onto gelatin-coated
slides. Sections were fixed in 4% formaldehyde, treated
with acetic anhydride, dehydrated and defatted.
Oligodeoxynucleotide probes (48 mer), kindly provided by
W. Scott Young, III and Michael Brownstein, were labelled
using 35S-dATP with terminal deoxytransferase.
Approximately 500,000 cpm of probe was applied to each
section and hybridization performed overnight at 37°. The
sections were washed sequentially in 50% formamide/2X SSC
at 40°, exposed to Kodak Ortho M film, and optical density
measured with an image analysis system using ^{35}S-standards.
CRH mRNA is expressed dpm/mg tissue based on these stand-
ards. Data represent the mean of 4 to 14 animals per
group = significantly different from LEW/N stress and
LEW/N and F344/N controls; $P=0,0002$ by AVOVA (f-TEST =
4.283, df 72) AND $P<0.05$ by Fisher PLSD. Control values
were not statistically significantly different from each
other.

DETAILED DESCRIPTION OF THE INVENTION

A single intraperitoneal injection of an aqueous
suspension of Group A Streptococcal cell wall fragments
(peptidoglycan-group specific polysaccharide) into LEW/N
female rates induces severe, rapid onset, acute thymic-
independent arthritis, followed by a chronic proliferative
and erosive thymic-dependent arthritis. However, histo-
compatible F344/N female rats, along with several other
histocompatible strains, develop only minimal, transient
swelling of the hind paws (1, 2). Strain dependent
differences in response to the cell walls are also noted
in the degree of splenic hypertrophy, blood leukocytosis,
and the development of hepatic granulomata. The develop-
ment of severe inflammatory pathology in the LEW/N rat is
not related to differences in the quantities, the sites of

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localization, or the duration of persistence of the cell wall fragments. In both LEW/N and F344/N rats, the cell walls localize to the spleen, liver, bone marrow and synovial blood vessels of the peripheral joints (1, 3, 4).

5   LEW/N rats develop a more persistent inflammatory reaction at the sites of cell wall localization than do F344/N rats (5). In vitro analyses of various mononuclear cell responses to cell walls have not provided fundamental insights into the mechanisms underlying the marked suscept-

10   ibility to streptococcal cell wall-induced disease in the LEW/N rats and resistance in F344/N rats, although small differences between strains have been noted (6-8, and Wilder et al, unpublished data).

Histologically, the earliest changes in the acute

15   phase arthritis in LEW/N rats are synovial microvascular injury associated with increased endothelial cell Ia expression. This is rapidly followed by infiltration of Ia positive macrophages in the synovium (1). At all time points F344/N rats exhibit little or no inflammation

20   compared to LEW/N rats. This difference in the degree of synovial macrophage and endothelial cell Ia expression, that parallels the development of arthritis, is the most striking immunohistological difference between the two strains. This difference is even observed in athymic

25   LEW.rnu/rnu and F344.rnu/rnu rats. This suggests that there may be a factor or factors regulating both Ia expression and the acute thymic-independent phase of SCW arthritis, and that the difference in arthritis suscepti- bility between the two strains may be related to the

30   presence of a down-regulator of Ia operative soon after injection of SCW in F344/N but not in LEW/N rats.

Corticosteroids are both potent endogenous down- regulators of Ia expression, and potent endogenous immuno- suppressive and anti-inflammatory agents (9-13). Corti-

35   costerone is released early in the course of inflammation through stimulation of the HPA axis by inflammatory media- tors such as endotoxin and interleukin-1 (IL-1) (14-23). Since SCW activate macrophages and stimulate release of

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IL-1, and are chemically related to endotoxin (bacterial lipopolysaccharide, LPS) (24), and since IL-1 is critical in maintaining the normal feedback loop between the immune system and central nervous system (CNS) (11-23, 25-26), the early ACTH and corticosterone responses to SCW and IL-1 alpha in inbred F344/N and LEW/N rats and outbred HSD rats were compared. Since serotonin (5-HT) is also released from platelets during inflammation, and down-regulates Ia expression (27, 28), and since 5-HT pathways represent another route of hypothalamic-pituitary stimulation (29-31), the effect of the serotonin agonist, quipazine, on acute ACTH and corticosterone responses in F344/N, LEW/N and HSD rats was also compared. Furthermore, to evaluate the direct involvement of glucocorticoids in the observed SCW susceptibility of LEW/N rats and SCW resistance of F344/N rats, the ability of replacement doses of glucocorticoids to suppress the SCW susceptibility of the former and the ability of a potent glucocorticoid antagonist RU 486 to reverse the SCW resistance of the latter was examined.

Animals: One hundred gram, virus antibody free, female, inbred F344/N and LEW/N rats, and outbred Harlan-Sprague-Dawley (HSD) rats, purchased from Harlan Sprague Dawley (Indianapolis, IN), were acclimatized to 12 hour on-12 hour off light cycles, prior to intraperitoneal injection of various inflammatory mediators.

Drugs and inflammatory mediators: Group A Streptococcal cell wall peptidoglycan-group specific carbohydrate (SCW) was prepared in phosphate buffered saline (PBS), as previously described (1). It was injected at a concentration of 0.02 to 2 mg of cell wall rhamnose per rat. Recombinant human interleukin-1 alpha (IL-1 alpha): IL-1 alpha (32) was a generous gift from Drs. P. Kilian and P. Lomedico, (Hoffman-LaRoche, Nutley, N.J.). It was injected at doses ranging from 0.1 to 5 μgm per rat. Specific activity ranged from 3×10^8 to 2.5×10^9 Units/μgm. 1 unit of IL-1 activity was defined in the D10 cell bioassay, as previously described (32). Endotoxin

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levels in final concentrations injected were less than
0.0013 EU/100 μl. Quipazine was purchased from Sigma
Chemical Company (St. Louis, MO). It was injected at
doses ranging from 0.1 to 5 mg per rat. Dexamethasone for
cell culture was purchased from Sigma Chemical Company
(St. Louis, MO), and used in doses ranging from 0.01 μg to
100 μgm per rat. RU 486: The glucocorticoid receptor
antagonist, RU 486, (33, Philibert, D., Deraedt, R., &
Teutsch, G. (1981) Proc. VIII International Congress of
Pharmacology, p. 668.) was a generous gift from Roussel-
UCLAF (Paris, France). It was suspended in sterile normal
saline for intraperitoneal (i.p.) injection, at doses
ranging from 0.03 mg to 3 mg per rat. LY53857: The
serotonin (5-hydroxytryptamine, 5-HT$_2$) antagonist, LY53857
(6-methyl-1-[1-methylethyl] ergoline-8-carboxylic acid, 2-
hydroxy-1-methylpropyl ester [Z]-2-butenedioate) (34), was
a generous gift from Dr. M. Cohen, Lilly Research Labora-
tories, Eli Lilly and Co. (Indianapolis, IN). Rat/human
corticotropin releasing hormone (CRH) was purchased from
Peninsula Laboratories (Belmont, CA), and was used at
doses ranging from 0.01 to 8 μgm per rat.

Hormone assays: Plasma corticosterone was
quantitated by radioimmunoassay (35) kit purchased from
Radioassay Systems Laboratories, Inc., Immunochem Corpora-
tion (Carson, CA). Adrenocorticotrophic hormone (ACTH)
levels were determined by radioimmunoassay, as previously
described (36). Rats were injected i.p. between 10 and 11
AM, and blood was collected from 30 minutes to 4 hours
post-injection, for plasma ACTH and corticosterone mea-
surements. Inter- and intra-assay control variability for
corticosterone was 1.2% and 3.4% respectively; inter- and
intra-assay control variability for ACTH was 8.0% and 2.8%
respectively.

Severity of arthritis: Severity of arthritis was
quantitated by articular index, performed by a single
blinded observer, as previously described (2). Briefly,
articular index is the sum of the severity of arthritis
(scale 0-4, 4 - most severe arthritis) of each of the

limbs. Maximum articular index is 16.

Data analysis: A minimum of 5 rats per experi-
mental group were studied, and experiments were repeated a
minimum of 3 times. Data shown are mean ± standard error
5   of the mean (S.E.M.) of each group. Experimental groups
were compared to vehicle treated controls and to each
other, and statistical significance between groups were
determined by unpaired Student t test. Total ACTH and
corticosterone released were calculated by integrating the
10   areas under the curves, using the trapezoid rule.

RESULTS

Thymic-Independent and Thymic-Dependent
Phases of LEW/N SCW Induced Arthritis

Figures 1A and 1B show the repeat of an earlier
15   experiment (1) in which the articular index (AI) was
assessed in euthymic versus athymic LEW and F344 rats
injected with SCW. Five animals in each group were
treated with a single intra-peritoneal dose of SCW at day
0, and observed from 6 weeks. The arthritis induced in
20   euthymic LEW/N rats is diphasic, with a rapid-onset acute
inflammatory component developing as early as 24 hours
after injection of SCW, and a later chronic component
developing at 3 to 6 weeks post-injection. Athymic
LEW.rnu/rnu rats do not develop the late phase arthritis,
25   but do develop the early inflammatory component and a
continue low grade chronic synovitis. The early phase of
SCW arthritis in LEW rats is, therefore, thymic-indepen-
dent, and the late phase is thymic-dependent. The very
small percentage of euthymic and athymic F344 rats that
30   develop mild arthritis develop only the early thymic-
independent component, which rapidly resolves. The pre-
sence of a strain difference in the acute, thymic-indepen-
dent phase of SCW arthritis in athymic LEW.rnu/rnu versus
F344.rnu/rnu rats indicates that the thymic-independent
35   phase of the arthritis is genetically regulated, and that
the regulating factor or factors are operative very early
in the disease.

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Corticosterone Responses to SCW, IL-1 Alpha and Quipazine in Outbred HSD Rats Versus Inbred F344/N and LEW/N Rats

Since, as discussed above, corticosterone is a potent down-regulator of Ia expression which is released

5   early in the course of inflammation through stimulation of the HPA axis by inflammatory mediators (14-23), the early ACTH and corticosterone responses to SCW, IL-1 alpha and the serotonin (5-HT) agonist, quipazine, in inbred F344/N and LEW/N rats and outbred HSD rats were compared.

10   Intraperitoneal SCW, IL-1 alpha, and the serotonin (5-HT) agonist quipazine all induced marked plasma corticosterone responses in F344/N rats at one hour post i.p. injection (Figures 2A through 2D and Table 1). In contrast, these agents induced only minimal (SCW, quipazine)

15   or absent (IL-1 alpha) plasma corticosterone responses in LEW/N rats (p<0.01). Outbred HSD rats exhibited mean corticosterone responses intermediate between the low LEW/N and high F344/N responses. Corticosterone responses of HSD rats showed a wide spread, and fell into two

20   groups: one overlapping the low LEW/N responses, and the other overlapping the high F344/N responses.

Table 1. Plasma corticosterone in PBS, SCW, IL-1 alpha or quipazine treated HSD, F344/N or LEW/N rats.

PLASMA CORTICOSTERONE, ng/ml (mean ± S.E.M.)

25

Strain Quipazine	PBS	SCW	IL-11 alpha	
HSD	162.6±33.4	656.1±44.6	389.4±54.1	656.8±46.3
F344/N	130.7±19.7	702.8±32.7	913.7±90.0	721.2±43.9
LEW/N	130.2±16.2	350.2±31.3	162.5±41.2	397.6±51.2

30

Data represents mean ± S.E.M. of plasma corticosterone shown in Figures 2A through 2D. Plasma corticosterone was

35   determined by radioimmunoassay of plasma collected 60 minutes post-i.p. injection of PBS, SCW (2 mg cell wall rhamnose/rat), IL-1 alpha (1 μgm/rat) or quipazine (1 mg/rat) in HSD, F344/N or LEW/N rats.

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The one hour time point of corticosterone measurement and doses of mediators used were those found to be associated with maximal corticosterone responses in time course and dose response experiments (Figures 3A through 3F and 4A through 4F).

Time Course Kinetics of Plasma ACTH and Corticosterone Versus Responses to SCW, IL-1 Alpha and Quipazine in LEW/N Versus F344/N Rats

Figures 3A through 3F show that while plasma ACTH peaked at 30 to 60 minutes post-injection in both F344/N and LEW/N rats, the LEW/N plasma ACTH response to SCW, IL-1 alpha and quipazine was consistently lower than the F344/N response at all time points. Similarly, the LEW/N plasma corticosterone response was lower than the F344/N response at all time points. Total ACTH and corticosterone secreted over the entire time course in response to SCW, IL-1 alpha, or quipazine was significantly less in LEW/N rats than in F344/N rats (Table 2). In F344/N rats, compared to LEW/N rats, plasma ACTH increased more than 3 fold as much in response to IL-1 alpha; more than 2 fold as much in response to SCW, and more than 1.6 fold in response to quipazine. F344/N rats increased plasma corticosterone more than 2 fold in response to SCW and IL-1 alpha, and 1.4 fold in response to quipazine when compared to LEW/N rats.

Table 2. Total plasma ACTH and corticosterone secreted over 4 years in response to SCW, IL-1 alpha or quipazine in LEW/N versus F344/N rats.

TOTAL ACTH (ng/mlx240 min)
(mean ± S.E.M.)

	F344/N	LEW/N	n	p	*F/L
SCW	94.5±4.7	40.3±1.8	20	<0.001	2.3
IL-1	64.9±5.4	20.4±3.7	23	<0.001	3.2
Quipazine	95.5±12.0	57.3±5.5	20	<0.05	1.6

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TOTAL CORTICOSTERONE, (μg/mlx240 min)
(mean ± S.E.M.)

SCW	184.9±3.9	70.3±11.9	32	<0.001	2.6
IL-1	104.5±3.7	38.9±7.9	33	<0.001	2.7
Quipazine	119.6±5.4	87.1±4.4	20	<0.01	1.4

*F/L = ratio of total ACTH or corticosterone secreted by F344/N (F) rats versus LEW/N (L) rats.

Data represent mean ± S.E.M. of total plasma ACTH and corticosterone secreted by F344/N versus LEW/N rats in response to i.p. SCW (2 mg cell wall rhamnose/rat), IL-1 alpha (1 μg/rat), or quipazine (1 mg/rat). Data were derived, using the trapezoid rule, by calculation of the area under time course curves shown in Figures 3A through 3F.

<u>Dose-Responses of Plasma ACTH and Corticosterone to SCW, IL-1 Alpha and Quipazine in LEW/N Versus F344/N Rats</u>

Figure 4A through 4F show the plasma ACTH and corticosterone responses of LEW/N versus F344/N rats treated with varying doses of SCW, IL-1 alpha or quipazine. At all mediator doses tested, LEW/N rats had lower plasma ACTH and corticosterone levels than F344/N rats.

<u>Plasma ACTH and Corticosterone Responses of LEW/N Versus F344/N Rats to Rat/Human CRH</u>

LEW/N ACTH and corticosterone responses to various doses of i.p. rat/human CRH were lower than F344/N responses (Figures 5A and 5B).

<u>Pituitary, Adrenal and Thymus Weights in F344/N Versus LEW/N Rats</u>

Pituitary weights, although not significantly different, were greater in F344/N compared to LEW/N rats (Table 3). F344/N adrenal gland weights were slightly but significantly greater than adrenal gland weights from age-matched LEW/N rats (p<0.01). LEW/N thymus weights were significantly higher than F344/N thymus weights (p<0.01), in age-matched rats.

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Table 3. Pituitary, adrenal and thymus weights in age-matched LEW/N and F344/N rats.

Strain Thymus Weight	(n)	Pituitary Weight	(n)	Adrenal Weight	(n)
		(mg) (mg)		(mg)	
F344/N 260.0±8.9	(10)	9.6±0.5	(27)	15.6±0.5	(10)
LEW/N 307.3±8.2	(13)	8.5±0.4	(26)	13.3±0.7	(10)
P value		N.S.		<0.01	<0.01

Inhibition of SCW Arthritis By Dexamethasone
Treatment of LEW/N Rats

Since corticosterone responses to SCW were clearly blunted in LEW/N rats, the effect of the corticosteroid, dexamethasone, on severity of arthritis in SCW treated LEW/N rats (Table 4) was evaluated. Five animals per group were injected i.p. with SCW (2 mg cell wall rhamnose/rat) on day 0, together with various doses of dexamethasone. Dexamethasone treatment was continued for 72 hours at doses ranging from the physiologic replacement range of 0.5 μg twice daily (b.i.d.) or 1 μg once daily (QD) to doses in the pharmacologic range, (10 - 100 μg QD). Not only did dexamethasone doses in the pharmacologic range totally suppress the arthritis induced by SCW, but doses in the physiologic range (1 μg QU or 0.5 μg b.i.d.) also significantly suppressed the severity of arthritis as determined by arthritis index (A.I.) compared to SCW plus saline treated controls (p<0.05).

Table 4. Dexamethasone suppression of SCW arthritis in LEW/N rats (72 hrs).

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Dexamethasone Dose (μg)	Incidence of Arthritis	Severity of Arthritis (mean A.I.±S.E.M.)
0.0	5/5	8.3±2.1
0.1 (b.i.d.)	5/5	8.4±1.8
0.5 (b.i.d.)	4/5	3.4±1.5*
1.0 (QD)	5/5	2.0±0.5*
10.0	0/4	.0*
100.0	0/5	.0*

*A.I. significantly less than A.I. of control animals (dexamethasone dose 0, saline only) (p<0.05). LEW/N rats were injected with a single dose of SCW (2 mg cell wall rhamnose/rat), followed by dexamethasone, at doses indicated, or saline controls. Dexamethasone injections were given once (QD) or twice (b.i.d.) daily for a total of 72 hours, and severity of arthritis (A.I., articular index) was quantitated at 72 hours post-SCW injection by a single blinded observer.

Effect of the Corticosterone Receptor Antagonist, RU 486, or the 5-HT$_2$ Antagonist, LY53857, on F344/N Rats Treated with SCW

Table 5 shows the effect or treatment of F344/N rats with SCW plus the corticosterone receptor antagonist RU 486, or SCW plus the 5-HT$_2$ antagonist LY53857, compared to either agent alone. Five animals per group were injected i.p. with SCW (2 mg cell wall rhamnose per rat) on day 0. Various doses of RU 486 were injected i.p. simultaneously with SCW, and the RU 486 was readministered once daily for up to 72 hours post-SCW injection LY53857 treatment was begun simultaneously with SCW and continued twice daily for 72 hours. Minimal mortality was observed in F344/N rats treated with SCW alone, and no mortality was observed in F344/N rats treated with RU 486 or LY53857 alone. Doses of RU 486 which had no effect alone (3) mg QD), were highly toxic when administered i.p. together with SCW, resulting in 100% mortality. Doses of RU 486 as

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low as 0.03 mg QD, when administered to SCW-treated rats,
were still associated with significant inflammatory
morbidity and mortality compared to controls. RU 486 has
previously been shown to exacerbate carrageenin-induced
5   inflammation, without significant mortality (33).
Increased mortality in the SCW-arthritis model was
probably related to the severe peritonitis which developed
in association with the combined i.p. administration of
the two agents. At doses of RU 486 low enough to permit
10   survival, surviving rats developed acute arthritis, in
some cases of moderate severity, e.g., mean A.I. = 4.5 at
0.3 mg RU 486 dose. Concurrent treatment of F344/N rats
with SCW and the 5-HT$_2$ antagonist LY53857 was not associ-
ated with significant mortality, but was associated with
15   development of mild to moderate arthritis compared to
control rats treated with either agent alone (p<0.05).
Although not all dosing variables were explored, the data
clearly show that blocking the effects of corticosterone
or 5-HT in SCW treated F344/N rats results in severe or
20   even fatal systemic inflammatory disease.

Table 5. Effects RU 486 or LY53857 on mortality and arthritis in F344/N rats treated with SCW

Agent(s) Injected Rats	Mortality	Incidence of Arthritis in Surviving Rats	Severity of Arthritis in Surviving (A.I.)
SCW + saline	2/15	1/13	0.2
saline + RU 486 3.0 mg	0/6	0/6	0
SCW + RU 486 0.03 mg	1/5*	2/4	0.5±0.3
SCW + RU 486 0.1 mg	2/5*	1/3	0.3±0.3
SCW + RU 486 0.3 mg	3/5*	1/2	4.5±4.5**
SCW + RU 486 1.0 mg	4/5*	0/1	0
SCW + RU 486 3.0 mg	5/5	-	-
saline - LY53857 0.3 mg	0/7	0/7	0
SCW + LY53857 0.03 mg	1/5	2/4	2±1.2**
SCW + LY53857 0.3 mg	0/5	2/5	2±1.5

*Surviving rats receiving RU 486 plus SCW showed ruffled fur and peritoneal inflammation at necropsy.

** $p < 0.05$ compared to articular index (A.I.) of SCW plus saline treated F344/N rats were treated with a single injection of SCW (mg cell wall rhamnose/rat), followed by daily i.p. injections of RU 486, or twice daily i.p. injections of LY53857, at doses indicated. Control animals were treated with SCW plus saline, RU 486 plus saline, or LY53857 plus saline. Articular index was quantitated by a blinded observer at 72 hours post-SCW injection. Maximum A.I. is 16.

F344/N rats' and LEW/N rats' levels of CRH, ACTH and corticosterone responses to behavior stress were compared as indicated in Figures 9A through 9F. The differential pituitary-adrenal responses were measured throughout the time course of exposure to restraint as indicated in tables 6 and 7.

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Table 6.   Total hypothalamic CRH content, and pituitary
ACTH content in F344/N and LEW/N rats exposed to restraint

	F344/N	LEW/N	p
total CRH content/hypothalamus (pg/hypothalamus)			
control	1706±153	2352±109	<0.05
3 hour restraint	2159±76	2436±66	<0.05
p	<0.05N.S.		
total ACTH content/pituitary (mg/pituitary)			
control	134.3±17.2	207.1±9.0	<0.05
3 hour restraint	115.0±10.2	216.8±14.6	<0.05
p	N.S.	N.S.	

Legend: Total hypothalamic CRH content and pituitary ACTH
content was measured in hypothalami and pituitaries from
LEW/N or F344/N rats restrained for three hours compared
to controls.   Hypothalami were excised and CRH and ACTH
quantitated after extraction, as previously described (3).
Data represent mean ± S.E.M. of 10 animals per condition.
Statistical significance was determined by ANOVA followed
by Duncan multiple range.

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Table 7. Plasma ACTH and corticosterone and behavioral responses to restraint and open field in F344/N, LEW/N and HSD rats

	F344/N	LEW/N	HSD
Restraint			
Plasma ACTH (pg/ml)			
control	135.7±9.6	180.5±14.2	170.5±13.9
3 hour restraint	231.5±21.5	157.6±25.4*	170.4±10.6*
Plasma corticosterone (ng/ml)			
control	20±8	15±5	24±6
3 hour restraint	332±104	19±7*	57±13*
Fecal boli			
3 hour restraint	6.4±1.1	1.3±0.5*	57.2±1.4
Open Field			
Outer squares crossed	39±5	52±2*	57±4.5*
Inner squares crossed	7.6±1	5.4±1.1	4.3±0.5*
Rears	6.4±1	4.6±0.6	3.8±0.6*
Fecal boli (open field)	0.7±0.4	0.7±0.4	0
Grooming	2.8±0.6	0.1±0.1*	0*

Legend: Plasma corticosterone and behavioral responses to restraint or open field testing. F344/N, LEW/N or HSD rats were either restrained for 3 hours, or exposed to an open field (5). Behavior in the open field was characterized by quantitation of numbers of outer and inner squares crossed, number of fecal boli produced, and number of grooming behaviors. Data represent mean ± S.E.M. of 10 animals per group. Experiments were performed in triplicate.* = significantly different from F344/N, $p, 0.05$, ANOVA followed by Duncan Multiple Range test.

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DISCUSSION

One of the earliest events that occurs in streptococcal cell wall injected LEW/N rats, and even in athymic nude LEW.rnu/rnu rats, is enhanced Ia expression on synovial endothelial cells. This develops concomitantly with the inflammatory process, and the intensity of expression parallels the severity of the arthritis. In marked contrast, insignificant enhancement of Ia antigen expression develops in SCW-injected euthymic and athymic F344 rats (1). Corticosteroids are both potent endogenous down-regulators of Ia expression and potent endogenous immunosuppressive and anti-inflammatory agents (9-13). In the experiments reported here, it has been shown that acute corticosterone responses to SCW IL-1 alpha and quipazine are severely depressed in arthritis susceptible, high Ia-expressing LEW/N rats, compared to arthritis resistant low-Ia expressing F344/N rats. Outbred HSD rats, which exhibit an intermediate mean susceptibility to SCW induced arthritis with wide vari-ability (2), also showed an intermediate mean and wide variability of corticosterone responses to these media-tors. Furthermore, replacement of corticosterone with physiologic doses of dexamethasone significantly sup-pressed the severity of SCW arthritis in LEW/N rats. Conversely, antagonism of corticosterone in F344/N rats, with the corticosterone receptor antagonist RU 486, was associated with increased mortality, exacerbation of inflammation, and development of mild to moderate acute arthritis in this otherwise resistant strain. It is clear from these studies that, whether present on a genetic basis, as in LEW/N rats, or on a pharmacological basis, as in RU 486 treated F344/N rats, a deficiency in the corti-costerone response to SCW is associated with development of susceptibility to SCW-induced inflammatory disease.

Elevation of corticosterone during inflammation results from stimulation of the hypothalamic-pituitary-adrenal (HPA) axis by inflammatory and immune mediators, such as endotoxin (bacterial lipopolysaccharide, LPS) and

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interleukin-1 (IL-1) (14-23). Since SCW (peptidoglycan-group specific polysaccharide) are chemically related to endotoxin, and stimulate the release of IL-1 from activated macrophages (24), SCW could increase corti-

5   costerone via IL-1 stimulation of the HPA axis. The studies presented here show that, in addition to depressed corticosterone responses to SCW, LEW/N rats have absent corticosterone responses to IL-1. Recent studies have shown that IL-1 stimulates the HPA axis at primarily the

10   hypothalamic level by inducing CRH release (15-23). LEW/N rats have depressed ACTH responses which parallel the low corticosterone responses to SCW and IL-1, in contrast to F344/N rats which have higher ACTH and corticosterone responses. These initial findings suggest that the HPA

15   axis defect in LEW/N rats is at either the hypothalamic and/or the pituitary level.

        The smaller adrenal glands and the larger thymuses of LEW/N compared to F344/N rats, are also consistent with deficient HPA axis responses and chronic mild hyposecre-

20   tion of corticosterone. LEW/N rats also have depressed ACTH and corticosterone responses to exogenous CRH. This could be secondary to inadequate priming of the anterior pituitary corticotroph by endogenous CRH or other ACTH secretagogues, or to some inherent defect of this cell.

25   It is impossible from the data presented here to precisely define the site of the defect, whether hypothalamic or pituitary, particularly since chronic over- or understimu-lation of the HPA axis, from whatever cause, could result in secondary changes in baseline and stimulated levels of

30   CRH, ACTH and corticosterone production, as well as secondary changes in adrenal and thymus sizes (13, 37). Further studies are required to determine the precise anatomical and molecular site of the lesion in LEW/N rats.

        The observation that LEW/N rats are deficient in

35   ACTH and corticosterone responses to the 5-HT agonist quipazine, as well as to IL-1 and SCW, suggests that the defect in these rats is not solely at the level of IL-1 stimulation of the HPA axis. The greater LEW/N

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corticosterone response to quipazine compared to IL-1 alpha may be related to the multiple pathways through which 5-HT and 5-HT agonists stimulate the HPA axis (31, 38-40), perhaps allowing quipazine to partially bypass the LEW/N defect in IL-1 - hypothalamic-pituitary pathways. This hypothesis is supported by existing data indicating that serotonin represents both a major CRH and a potent ACTH secretagogue (31, 41, 42). The importance of 5-HT pathways in intactness of the inflammatory mediator - HPA axis loop and arthritis resistance is also suggested by the association of arthritis with LY53857 treatment of SCW-injected rats.

The greater LEW/N corticosterone and ACTH response to SCW compared to IL-1 may also be related to stimulation of the HPA axis at multiple levels by the many inflammatory mediators released by SCW, including IL-1, interleukin-2 (IL-2), tumor necrosis factor (TNF) and 5-HT. Although 5-HT does not cross the blood brain barrier, 5-HT released during inflammation could hypothetically directly stimulate pituitary release of ACTH (31, 41, 42). The physiologic relevance of such a potential route of 5-HT stimulation of the HPA axis is, however, not clear, since it would be dependent on adequate systemic concentrations of 5-HT reaching central sites.

Taken together, the experiments reported here show that LEW/N rats represent a strain of rats genetically deficient in ACTH and corticosterone responses to several inflammatory or stress mediators, including SCW and IL-1, and 5-HT agonist, quipazine, and exogenous CRH. In contrast, F344/N rats represent a histocompatible, relatively SCW arthritis-resistant strain with intact, potent ACTH and corticosterone responses to the same inflammatory or stress mediators. Responses of HSD rats represent those of an outbred population, covering a wide range of both inflammatory mediator-HPA axis ACTH and corticosterone responses and SCW-arthritis susceptibility.

LEW/N and F344/N rats, therefore, represent a unique animal model for a genetically determined defect in

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the CNS - inflammatory/immune system feedback loop.
Whether of hypothalamic or pituitary origin, this defect
is associated with increased susceptibility to arthritis
in response to SCW, and could also contribute to the
increased susceptibility to other experimentally-induced
inflammatory diseases observed in LEW/N rats (43-50). The
data, coupled with the markedly enhanced inflammatory
disease in SCW-injected F344/N rats following
pharmacologic interruption of the HPA axis, and suppres-
sion of arthritis severity in SCW-injected LEW/N rats
following replacement doses of dexamethasone, provide
strong evidence that arthritis susceptibility in the LEW/N
rat, and resistance in the F344/N rat is regulated, at
least partially, through corticosterone production and HPA
axis responsiveness to inflammatory and possibly other
stress mediators.

The data may also have implications for
susceptibility to rheumatoid arthritis in humans. Rheuma-
toid arthritis is associated with a class II major histo-
compatibility complex (MHC) epitope that is shared amongst
several different haplotypes. Studies examining the
contribution of class II MHC (Ia) type to rheumatoid
arthritis susceptibility have suggested that MHC type and
sequence are only partially responsible for susceptibility
to rheumatoid arthritis (51). Another, as yet undefined
factor, perhaps one controlling regulation of Ia expres-
sion, may therefore contribute to susceptibility to
rheumatoid arthritis. The data presented here are consis-
tent with the concept that the additional factors regulat-
ing both Ia expression and susceptibility to arthritis are
corticosteroids and responsiveness of the HPA axis to
inflammatory and possibly other stress mediators. Several
other lines of evidence support this concept. Rheumatoid
patients are exquisitely sensitive to the disease-sup-
pressing effects of low doses of corticosteroids; rheuma-
toid arthritis frequently remits during hypercortisolemic
states, such as pregnancy, and exacerbates during hypo-
cortisolemic states such as the post-partum period. These

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observations, coupled with our data, suggest that evalua-
tion of hypothalamic-pituitary-adrenal axis responsiveness
to inflammatory and possibly other stress mediators, in
patients with rheumatoid arthritis, may provide new
5   insights into the disease process.

          Additional evidence indicates that susceptibility
to streptococcal cell wall (SCW)-induced arthritis in the
Lewis (LEW/N) rat, a model for human rheumatoid arthritis,
is due, in part, to defective inflammatory and stress
10   mediator-induced activation of the hypothalamic-pituitary-
adrenal axis.   To explore the mechanism of this defect,
the functional integrity of the hypothalamic corticotropin
releasing hormone (CRH) neuron in LEW/N rats was examined
and compared to histocompatible, arthritis-resistant
15   F344/N rats.   In response to SCW or recombinant inter-
leukin-1 alpha (rIL-1 alpha), LEW/N rats showed profoundly
deficient paraventricular nucleus CRH mRNA levels, hypo-
thalamic CRH content and CRH release from explanted
hypothalami in organ culture.   These data provide strong
20   evidence that the defective LEW/N ACTH and corticosterone
responses to inflammatory and other stress mediators, and
LEW/N susceptibility to experimental arthritis, are due in
part to a hypothalamic defect in the synthesis and secre-
tion of CRH.   The additional finding of deficient expres-
25   sion in LEW/N rats of the hypothalamic enkephalin gene,
which is coordinately regulated with the CRH gene in
response to stress, suggests that the primary defect is
not in the CRH gene, but is rather related to its inappro-
priate regulation.

30   We have found that, compared to F344/N rats, LEW/N
rats' CRH, ACTH and corticosterone responses to behavioral
stresses were profoundly blunted, consistent with their
severely defective CRH, ACTH and corticosterone responses
to inflammatory mediators.   Hence, LEW/N rats secreted
35   significantly less ACTH and corticosterone than did F344/N
rats in response to all stressors, i.e., novel environ-
ment, swim, restraint and ether (Figures 1A and 1B).
These differential pituitary-adrenal responses were

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maintained throughout the time course of exposure to
restraint (3 hour plasma ACTH: LEW/N = 170 ± 10 pg/ml;
F344/N = 232 ± 20 pg/ml; p<0.05; 3 hour plasma corti-
costerone: LEW/N = 19 ± 7 ng/ml; F344/N=332 ± 100 ng/ml,
5   p<0.05). However, by three hours in response to ether,
the differences in plasma ACTH and corticosterone levels
in the two strains tended to narrow (3 hour plasma ACTH:
LEW/N = 269 ± 8 pg/ml; F344/N = 377 ± 46 pg/ml p<0.05; 3
hour plasma corticosterone: LEW/N = 414 ± 12 ng/ml;
10   F344/N = 522 ± 36 ng/ml, p = N.S. The relatively greater
corticosterone response to swim stress and ether in LEW/N
rats probably reflects the greater relative potency of
these stressful stimuli compared to open field and re-
straint stresses. Furthermore, ether is known to activate
15   the HPA axis through both central pathways and direct
stimulation of pituitary corticotroph cells as well.

Consistent with the differential plasma ACTH and
corticosterone responses to physical restraint, LEW/N rats
failed to show any increase in CRH mRNA expression in the
20   PVN of the hypothalamus in contrast to F344/N rats in
which hypothalamic CRH mRNA expression increased signi-
ficantly during restraint. These findings are consistent
with our previous data showing deficient LEW/N plasma ACTH
and corticosterone responses and PVN CRH mRNA expression
25   in response to inflammatory mediators such as SCW, IL-1
alpha or quipazine, and suggest that hypothalamic CRH
biosynthesis in LEW/N rats, compared to F344/N rats, is
blunted in response to a wide range of stressful stimuli,
from chemical inflammatory mediators to behavioral stress
30   stimuli. Also consistent with their plasma ACTH and
corticosterone responses to ether, LEW/N CRH mRNA levels
in the PVN were increased over basal levels. These
findings demonstrate that during exposure to a powerful
stress stimulus, such as ether, the defect in CRH biosyn-
35   thesis in LEW/N rats can be enhanced, but still not to the
level observed in F344/N rats.

In response to intraperitoneal injections of Group
A streptococcal cell wall peptidoglycan-polysaccharide

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(SCW), inbred Lewis (LEW/N) female rats develop severe proliferative and erosive arthritis which mimics human rheumatoid arthritis. Histocompatible Fischer (F344/N) rats, on the other hand, do not develop arthritis in response to the same SCW stimulus (1-8). In light of recent postulated evidence that inflammatory mediator-activation of glucocorticoid secretion is one mechanism by which the immune response is appropriately regulated and restrained pituitary-adrenal responsiveness to SCW and other inflammatory stimuli in LEW/N and F344/N rats was previously explored (23, 52, 53). It was found that LEW/N rats have defective HPA axis responses to inflammatory and other stress mediators and that the response of F344/N rats to the same stimuli is intact, or above normal. Specifically, LEW/N rats, in contrast to F344/N rats, have markedly impaired plasma ACTH and corticosterone responses to intraperitoneally-injected SCW, to recombinant human interleukin-1 alpha (rIL-1 alpha), to the serotonin agonist, quipazine, and to synthetic rat/human corti-cotropin releasing hormine (CRH). In addition, LEW/N rats, compared to F344/N rats, have smaller adrenal glands and larger thymuses, consistent with chronic lack of stimulation by ACTH and corticosterone, respectively. Furthermore, arthritis and severe inflammation can be induced in otherwise SCW arthritis - resistant F344/N rats, by interruption of the HPA axis at its effector end-point, with the glucocorticoid receptor antagonist, RU 486. Taken together, these data indicate that LEW/N rats' pituitary and adrenal hyporesponsiveness to inflammatory and other stress mediators is a major factor contributing to their susceptibility to SCW arthritis and other experi-mental inflammatory diseases (43-50).

Pituitary ACTH hyporesponsiveness to stimuli can be primary, or secondary to lack of hypothalamic stimulation (36). In order to determine whether the impaired ACTH and corticosterone responses of LEW/N rats were hypothalamic in origin, the ability of streptococcal cells walls (SCW) or rIL-1 alpha to affect _in vivo_ hypo-

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thalamic CRH secretion in LEW/N and F344/N rats was
compared. Results of these studies show that the LEW/N
HPA axis defect involves the hypothalamus. In contrast to
F344/N rats, neuronal synthesis and secretion of CRH
within the PVN was markedly impaired.

The influence of inflammatory mediators on hypo-
thalamic expression of CRH mRNA was quantitated by in situ
hybridization with a CRH probe in PVN sections from F344/n
and LEW/N rats injected intraperitoneally with either SCW
or rIL-1 alpha. 80-100 gm virus antibody-free, F344/n and
LEW/N female rates (Harlan Sprague Dawley, Indianapolis,
IN) were injected intraperitoneally with SCW (2 mg cell-
wall rhamnose per 100 gm rat), or recombinant interleukin-
1 alpha (rIL-1 alpha, 1 μgm/100 gm rat). Recombinant IL-1
alpha was a kind gift from Drs. P. Kilian and P. Lomedico,
Hoffman-LaRoche, Nutley, NJ. Blood was immediately
collected and used to determine plasma corticosterone and
ACTH levels (35, 36). The brains were removed, and 4 mm
coronal slices containing the PVN were
frozen and stored at -80°C until 12 μm frozen sections
were cut and thaw-mounted onto twice gelatin-coated
slides. They were then processed for in situ hybridiza-
tion with CRH or enkephalin probes and analyzed as previ-
ously described (54, 55, 56). The hybridizations were
performed at 37°C for 20-24 hours in 600 mM Tris-HCl (pH
7.5), 50% formamide, 4 mM EDTA, 0.1% sodium pyrophosphate,
0.2% SDS, 0.2 mg/ml heparin sulfate, and 10% dextran
sulfate. The probes had specific activities of 10-15,000
Ci/mmol. Between 4 to 7 hours after injection of SCW, CRH
mRNA levels increased significantly in F344/N PVN, but did
not increase in LEW/N PVN (Figure 6A). This lack of a CRH
biosynthetic response to SCW in LEW/N rats could be
secondary to a defect in the CRH gene or in steps leading
to its activation. Coordinate regulation of the CRH and
enkephalin genes in the PVN after application of two
different stresses has been previously described (57, 58).
In order to determine whether induction of the enkephalin
gene by SCW was also defective in LEW/N rats, adjacent

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sections from the animals treated with SCW were examined for enkephalin expression by _in situ_ hybridization. The response was much greater in F344/N PVN than in the LEW/N PVN (Figure 6B). These results suggest that a common pathway that activates the CRH and enkephalin genes in the PVN in F344/N and other normal rats (57, 58) is defective in LEW/N rats.

Bacterial endotoxin lipopolysaccharide, (components of SCW) and IL-1 stimulate the HPA axis (14-22, 59). Since SCW induce IL-1 release (24), IL-1 is one probable mediator of SCW HPA axis stimulation. Indeed, rIl-1 alpha did induce significant (P<0.01) increases in plasma corticosterone and ACTH in F344/N rats, compared to LEW/N rats (F344/N ACTH = 480 pg/ml, LEW/N ACTH = 70pg/ml; F344/N corticosterone = 488 ng/ml, LEW/N corticosterone = 78 ng/ml, at one hour post-injection) (See preceding discussion). A similar single intraperitoneal injection of rIL-1 alpha (1 microgram per rat) however, did not significantly increase CRH mRNA or enkephalin mRNA in the PVN over baseline in either strain (Figures 6C and 6D). This discrepancy between the ability of rIL-1 alpha to augment plasma ACTH and corticosterone, and its inability to augment CRH mRNA levels in the PVN in F344/N rats, suggests that rIL-1 alpha, at the dose used, stimulates secretions, but not transcription of CRH. Alternatively, any increase in transcript levels induced by rIL-1 alpha may be below the level of sensitivity of the _in situ_ hybridization assay. The increase of CRH mRNA levels induced by SCW, in contrast to the lack of CRH mRNA response to rIL-1 alpha, could be related to two mechanisms; the more sustained nature of the SCW stimulus compared to rIL-1 alpha, or the multiple factors released by SCW, which should stimulate the CRH neuron via multiple pathways.

Total hypothalamic CRH content reflects the balance between CRH synthesis and secretion. Four hours after intraperitoneal injection of LEW/N or F344/N rats with SCW (2 mg cell wall rhamnose per rat), recombinant

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IL-1 alpha (1 μgm per rat), or phosphate buffered saline
(PBS, sterile, endotoxin free, GIBCO, Grand Island, NY),
rats were decapitated, and hypothalami were rapidly
removed, quick-frozen on dry ice and extracted. Total
immunoreactive CRH (iCRH) was quantitated by radioimmuno-
assay, as previously described (60). Hypothalamic
immunoreactive CRH (iCRH) content, measured 4 hours after
intraperitoneal injection of SCW, rIL-1 alpha, or phos-
phate buffered saline (PBS) is shown in Figures 7A and 7B.
In F344/N rats, hypothalamic content of ICRH increased
more than two fold over controls in response to either
intraperitoneal SCW or rIL-1 alpha. iCRH content in PBS-
injected and untreated animals was not significantly
different. In contrast, iCRH content did not change in
LEW/N rats injected with PBS, SCW or rIL-1 alpha, compared
to untreated LEW/N rats. The lack of change in hypotha-
lamic iCRH content of LEW/N rats in response to _in vivo_
administration of SCW or rIL-1 alpha is consistent with
the LEW/N rats' defective response of CRH mRNA to these
mediators. The SCW-induced increase in hypothalamic iCRH
in F344/N rats is consistent with their ability to in-
crease CRH mRNA levels in response to SCW. rIL-1 alpha's
capacity to increase CRH mRNA levels in response to SCW.
rIl-1 alpha's capacity to increase hypothalamic iCRH
content in F344N rats, but not CRH mRNA levels in the PVN
of these rats, suggests that IL-1 alpha may increase the
rate or efficiency of CRH mRNA translation and/or post-
transnational processing without causing detectable
increases in CRH transcript levels.

    In order to determine whether IL-1 stimulation of
CRH secretion is also defective in LEW/N rats, the ability
of rIL-1 alpha to induce iCRH release _in vitro_ from LEW/N
versus F344/N hypothalamic explants was compared. Hypotha-
lamic explants, obtained from untreated age-matched F344/N
or LEW/N rats, were cultured in the presence of various
concentrations of rIL-1 alpha and release of iCRH into the
culture supernate was quantitated by radioimmunoassay.
Hypothalamic explants were rapidly removed from untreated

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rats, as previously described (61, 62). The explants were incubated overnight at 37°C, 5% CO_2, in medium 199, (M199, GIBCO, Grand Island, NY), with 0.1% bovine serum albumin (BSA, grade V, Sigma Chemicals, St. Louis, MO). The hypothalami, in 48 well tissue culture plates, were then serially transferred every 20 minutes through a series of six wells containing one of the following additives, in order: control M199 (3 wells, total 60 minutes); M199 plus recombinant IL-1 alpha (10^{-13}M to 10^{-6}M; 2 wells, total 40 minutes); or 60 mM potassium chloride (1 well, 20 minutes). Immunoreactive CRH (iCRH) was assayed directly in the media, by a sensitive radioimmunoassay, as previously described (63). Only results from viable hypothalami, represented by those with a $\geq$ 90% iCRH response to 60 mM KCl over basal values, were included in the analyses. Figure 8 shows that rIL-1 alpha (10^{-13}M to 10^{-6}M induced a 150% increase in iCRH secretion over baseline from F344/N hypothalami, and no increase in iCRH secretion over baseline from LEW/N hypothalami.

As discussed previously, the LEW/N rats' defective ACTH and corticosterone responsiveness to inflammatory and other stress mediators is one critical factor in their susceptibility to SCW-induced arthritis. Our current findings suggest that the deficient LEW/N ACTH and corticosterone responses and associated susceptibility to arthritis are related to a lack of hypothalamic synthesis and secretion of CRH, and perhaps other stress hormones, in response to inflammatory and other stress mediators. The coordinate defect in LEW/N enkephalin mRNA synthesis in response to SCW provides evidence at the LEW/N rats' CRH biosynthetic defect is not specific to the CRH gene, but may result from a defect in its regulation. The present findings suggest a unique model for a mammalian autoimmune disease, in which a central nervous system defect results in an illness characterized by inadequate immune/inflammatory counter-regulation. Such a mechanism may also be relevant to human illnesses such as rheumatoid arthritis.

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Arginine Vasopressin Stimulation Test:

There are currently two methods available to test the integrity of HPA axis responses in humans: the CRH stimulation test, and the arginine vasopressin (AVP) stimulation test (Meller, W.H. et al, J. of Psychiatric Research, 21, No. 3, 269-277 (1987). Further details of this test can be found in NIH Clinical Project no. 87-M-85a which is hereby incorporated by reference. Both these methods have been developed at the NIH Clinical Center, and have been extensively studied in diseases such as depression and Cushings' syndrome. The CRH stimulation test determines sensitivity of ACTH and cortisol responses to exogenous CRH, and therefore tests the integrity of pituitary corticotroph cells' responses to stress. Since defective pituitary responsiveness to stress could be primary, or could be secondary to long-term hypothalamic defects as well, the CRH test also indirectly tests the integrity of hypothalamic responses to stress. A more direct measure of the integrity of hypothalamic CRH responses to stress is the AVP stimulation test. AVP synergizes with CRH in stimulating ACTH release. Decreased endogenous CRH would therefore result in decreased ACTH and cortisol secretion in response to exogenous AVP. Maximal ACTH and cortisol responses to AVP stimulation occur at 9:00 a.m., when CRH should be at its peak. Maximal ACTH and cortisol responses to CRH stimulation occur when CRH is at its nadir, at 4:00 p.m. An intravenous infusion of arginine vasopressin of 1.0 mIU/kg/min administered over a one hour period between 9:00 and 10:00 a.m. has been shown to have minimal side effects, and to maximally synergize with endogenous CRH in stimulating ACTH release. However, the infusion could be given at 8:00 to 9:00 p.m., depending on results of preliminary testing.

AVP Stimulation Test:

Arginine vasopressin will be administered by an intravenous infusion of 1.0 mIU/kg/min of arginine vasopressin over a one hour period between 9:00 a.m. and

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10:00 a.m. The infusions will be by means of a digitally controlled Extracorporeal Constant Infusion Pump, Model 2100. If any given dosage side effects such as nausea or gastrointestinal cramping occur, the infusion will be

5   stopped immediately. Upon cessation of side effects, the infusion may again commence at a dose established to be free of side effects from a given individual. Side effects should be rare even at the maximal doses proposed for this study. In addition to the standard intracath

10   placed for infusion of arginine vasopressin, blood will be drawn through a scalp vein needle in the contralateral arm, every 15 minutes before, during, and for two hours after the infusion. Eight cc's will be taken for every sample, and a total of 104 cc's will be taken for each

15   infusion. Blood will be assayed for ACTH and cortisol, and in some cases also for other hormones such as beta-endorphin, growth hormone, prolactin and oxytocin.

Synthetic arginine vasopressin will be obtained from the Parke-Davis Company. Park-Davis markets a highly

20   purified synthetic peptide with a sequence of naturally occurring arginine vasopressin and the commercial designa-tion Aqueous Pitressin.

The components for use by the means described herein can be assembled as kits for use in accord with the

25   teachings of the application. Such kits may contain the hypothalamic-pituitary-adrenal (HPA) axis stimulating drug, tracers such as I125 or an ELISA tracer, reagents specific for use in the assay chosen, antibodies to HPA axis hormone, and, our use as controls, standardized

30   plasma. Any of the HPA axis hormones such as GABA agonists/antagonists, MAO inhibitors, Dopamine uptake inhibitors/releasers, Cholinergic agents, serotonin agonists/releasers/uptake inhibitors, adrenergic agonists/antagonists/uptake inhibitors named at page 5 are

35   appropriate for use in kits in accord with the teachings of this disclosure. 1-metachloro-phenyl-piperazine (mCPP) is a particularly preferred stimulant.

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WHAT IS CLAIMED IS:

1. A method for testing the susceptibility of a mammal to inflammatory diseases which comprises the steps of: Administering to a mammal a compound which is effective in stimulating the hypothalamic-pituitary-adrenal axis; and measuring the level of hormones secreted by the pituitary or adrenal glands of said mammal.

2. The method of claim 1, which comprises the steps of administering to a mammal a compound selected from the group consisting of cytokines, growth hormones, neuroendocrine hormones such as corticotropin releasing hormone or arginine vasopressin (AVP), biogenic amines, agonists of biogenic amines, analogs of biogenic amines, monoamine oxidase inhibitors and biogenic amine uptake inhibitors; and measuring the level of glucocorticoids or adrenocorticotropic hormone in the blood plasma of said mammal.

3. The method of claim 1, wherein said inflammatory disease is arthritis, uveoretinitis, pneumonitis, encephalomyelitis, multiple sclerosis and hepatic granulomatas.

4. The method of claim 1, wherein said compound is interleukin-1, serotonin, corticotropin releasing hormone, AVP or quipazine and said hormone is cortisol, corticosterone or adrenocorticotropic hormone.

5. The method of claim 1, wherein the level of said hormones secreted by the pituitary and adrenal glands are measured 10 minutes to 4 hours after administration said compound.

6. A method for testing the susceptibility of the mammal to arthritis which comprises the steps of: Administering to a mammal an amount of interleukin-1 or quipazine effective to stimulate the hypothalamic-pituitary-adrenal axis; and measuring the level of adrenocorticotropic hormone or corticosterone in the plasma of said mammal between 10 minutes and 4 hours after administration of said interleukin-1 or quipazine.

7. The method of claim 6, wherein said mammal is

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a laboratory animal.

   8.   The method of claim 6, wherein said mammal is
a human.

   9.   A method for treatment of inflammatory
diseases which comprises the step of: administering to a
patient suffering from an inflammatory a compound which
stimulates the hypothalamic-pituitary-adrenal axis in an
amount effective to stimulate said axis.

   10.   The method of claim 9, wherein said compound
is administered to a patient suffering from rheumatoid
arthritis.

   11.   A kit comprising: (1) a HPA axis stimulating
agent; and (2) a means of measuring the HPA axis hormone
response in the patients's plasma.

   12.   A kit of claim 11 having, additionally, a
tracer.

   13.   A kit of claim 11 containing antibodies to
the HPA axis hormone.

   14.   A kit of claim 11 wherein the means of
measuring the HPA axis hormone response is an ELISA test.

   15.   A kit of claim 14 wherein the kit contains an
ELISA tracer.

   16.   A kit of claim 11 wherein the stimulating
agent is a GABA agonist/antagonist.

   17.   A kit of claim 11 wherein the stimulating
agent is 1-metachloro-phenyl-piperazine.

   18.   A kit of claim 11 containing therein a HPA
axis stimulating agent; an ELISA tracer, antibodies to HPA
axis hormone, and standardized plasma as a control.

   19.   A kit of claim 11 containing $^{I}125$ as a trac-
er.

   20.   The use of a compound to test the suscepti-
bility of a mammal to inflammatory diseases which
comprises the steps of: Administering to a mammal an
effective amount of a compound which stimulates the hypo-
thalamic-pituitary-adrenal axis; and measuring the level
of hormones secreted by the pituitary or adrenal glands of
said mammal.

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21.   The use of claim 20, which comprises the steps of administering to a mammal an effective amount of a compound selected from the group consisting of cyto-kines, growth hormones, neuroendocrine hormones such as corticotropin releasing hormone or arginine vasopressin (AVP), biogenic amines, agonists of biogenic amines, analogs of biogenic amines, monoamine oxidase inhibitors and biogenic amine uptake inhibitors; and measuring the level of glucocorticoids or adrenocorticotropic hormone in the blood plasma of said mammal.

22.   The use of claim 20, wherein said inflammatory disease is arthritis, uveoretinitis, pneumonitis, encephalomyelitis, multiple sclerosis and hepatic granulomatas.

23.   The use of claim 20, wherein said compound is interleukin-1, serotonin, corticotropin releasing hormone, AVP or quipazine and said hormone is cortisol, corticosterone or adrenocorticotropic hormone.

24.   The use of claim 20, wherein the level of said hormones secreted by the pituitary and adrenal glands are measured 10 minutes to 4 hours after administration said compound.

25.   The use of a compound to test for the susceptibility of a mammal to arthritis which comprises the steps of: Administering to a mammal an effective amount of interleukin-1 or quipazine which stimulates the hypothalamic-pituitary-adrenal axis; and measuring the level of adrenocorticotropic hormone or corticosterone in the plasma of said mammal between 10 minutes and 4 hours after administration of said interleukin-1 or quipazine.

26.   The use of claim 25, wherein said mammal is a laboratory animal.

27.   The use of claim 25, wherein said mammal is a human.

28.   The use of a compound to treat inflammatory diseases which comprises the step of: administering to a patient suffering from an inflammatory disease, a compound which stimulates the hypothalamic-pituitary-adrenal axis

in an amount effective to stimulate said axis.

FIG. 1B

FIG. 1A

FIG. 2A   FIG. 2B   FIG. 2C   FIG. 2D

CORTICOSTERONE (ng/ml)

SCW

FIG. 3B

TIME (minutes)

ACTH (pg/ml)

SCW

FIG. 3A

TIME (minutes)

FIG. 3D

FIG. 3C

FIG. 3F

FIG. 3E

FIG. 4A

FIG. 4B

FIG. 4C

FIG. 4D

FIG. 4F

FIG. 4E

FIG. 5A

FIG. 5B

FIG. 6A


FIG. 6B

rIL-1α

CRH
(copies x 10^4)

FIG. 6C

ENKEPHALIN
(copies x 10^3)

TIME (hr)

FIG. 6D

FIG. 7B

HYPOTHALAMIC iCRH
CONTENT
(pg/mg PROTEIN)

FIG. 7A

FIG. 8

% iCRH RELEASE OVER BASELINE

IL-1α CONCENTRATION (log Molar)
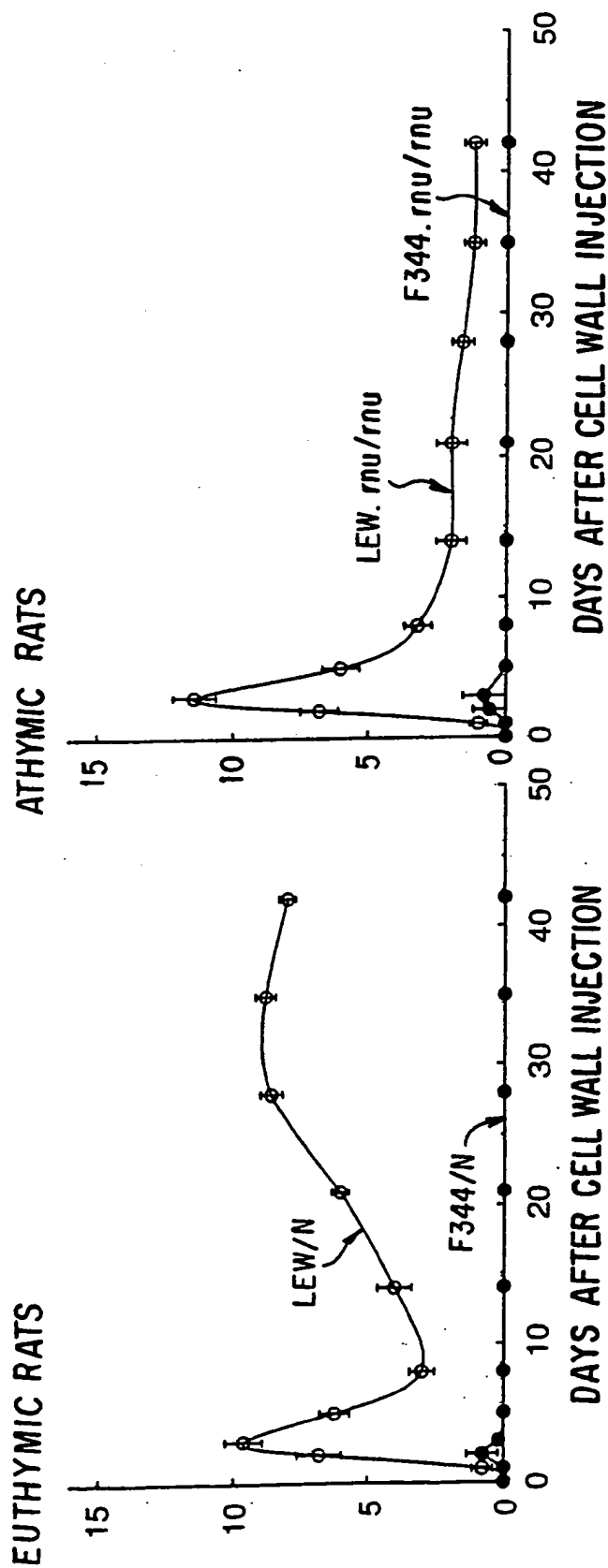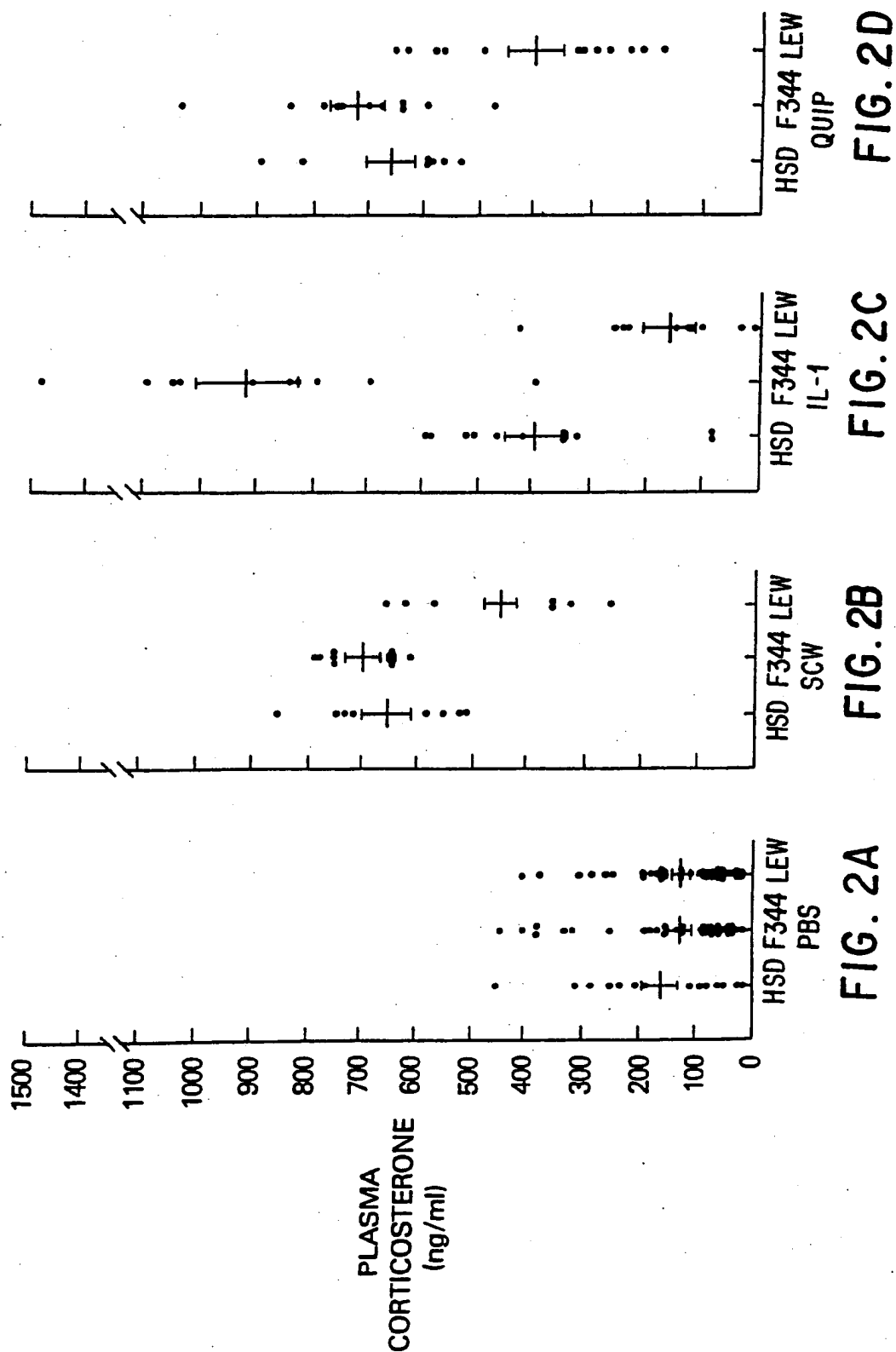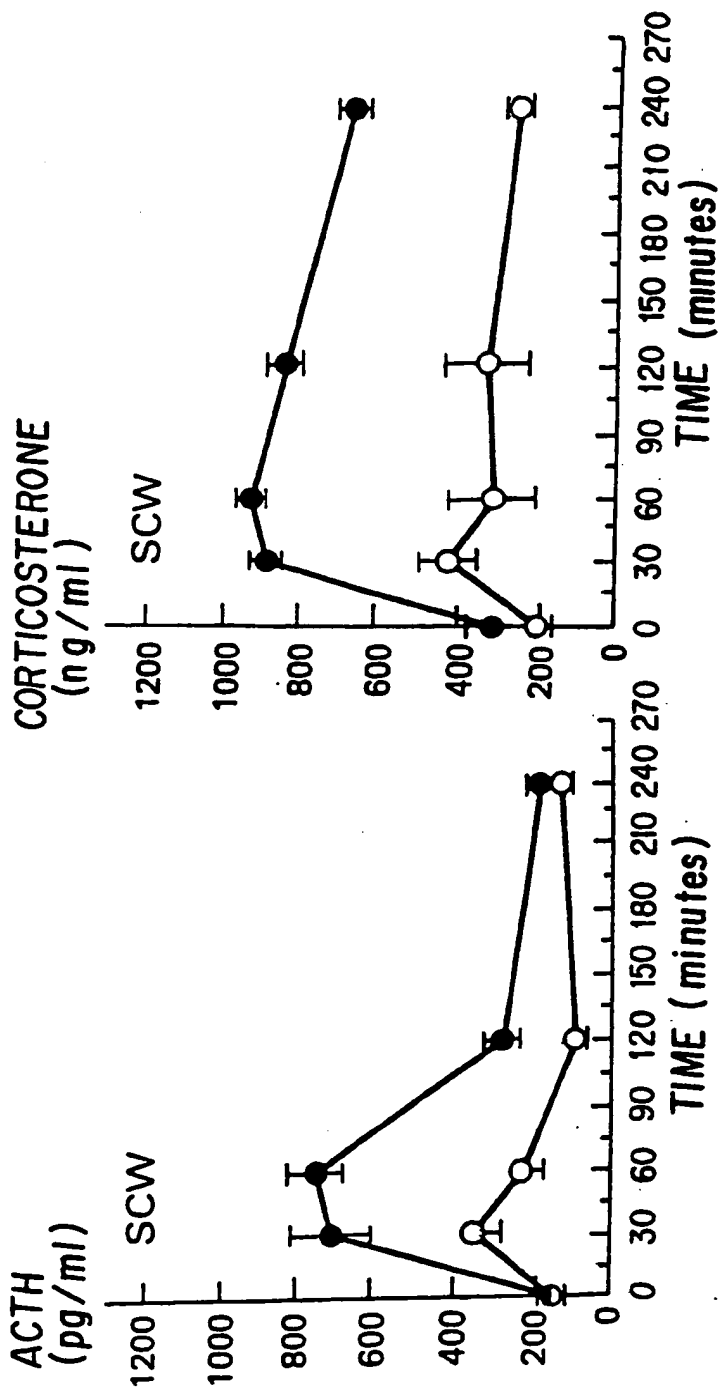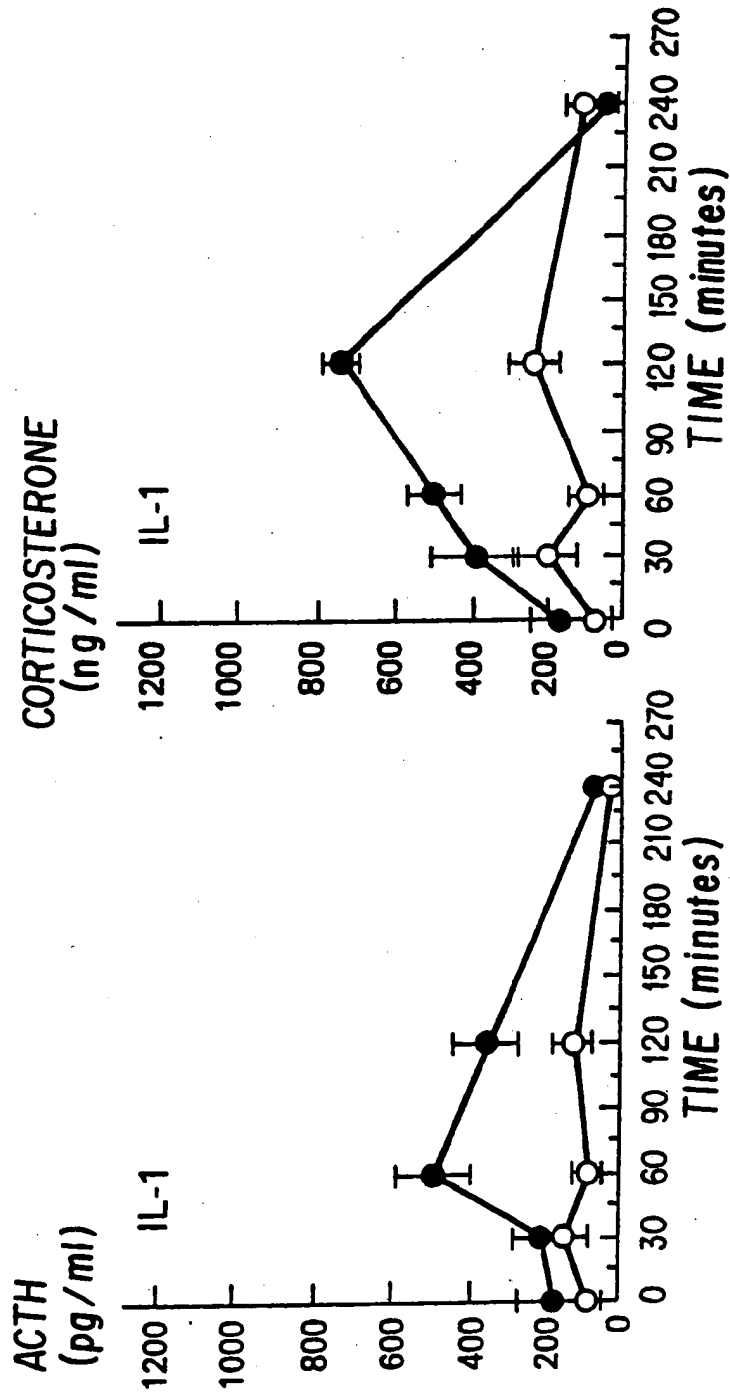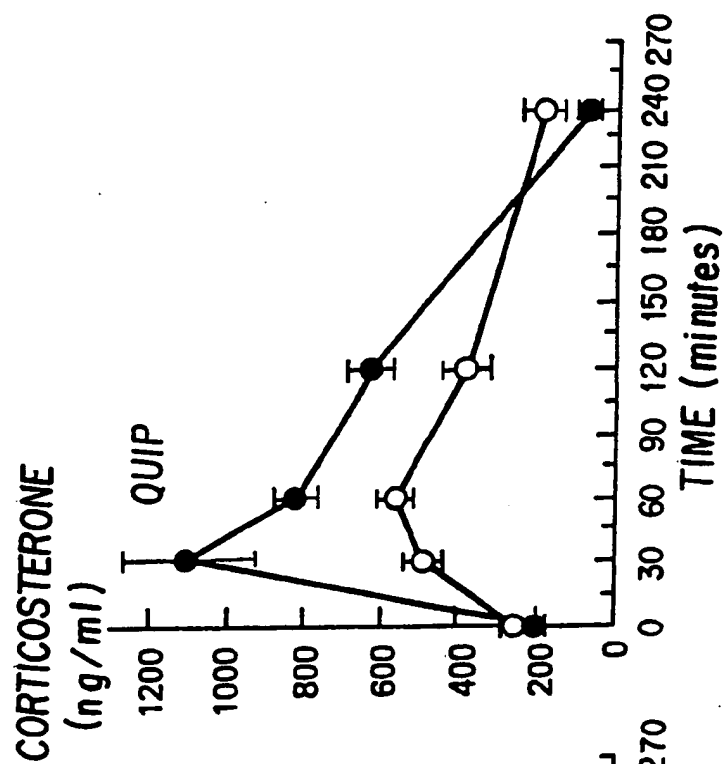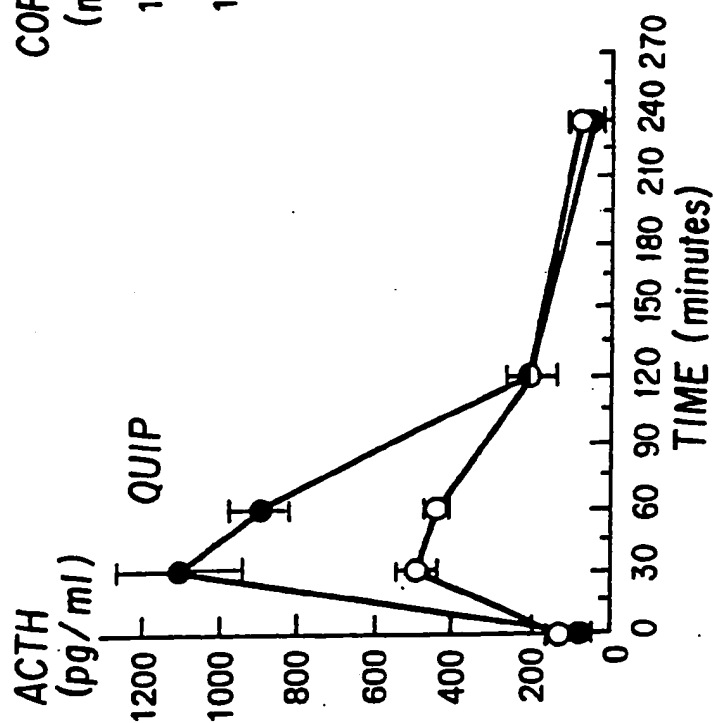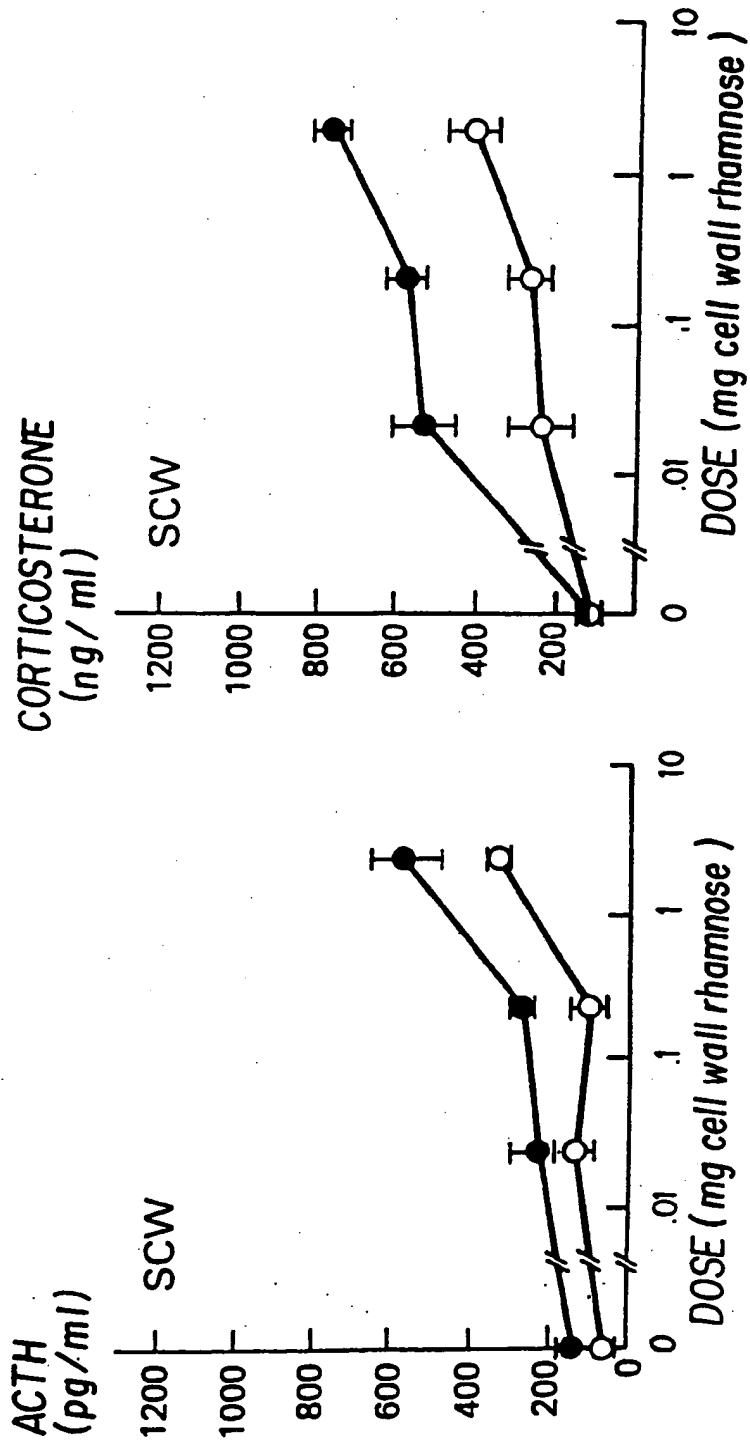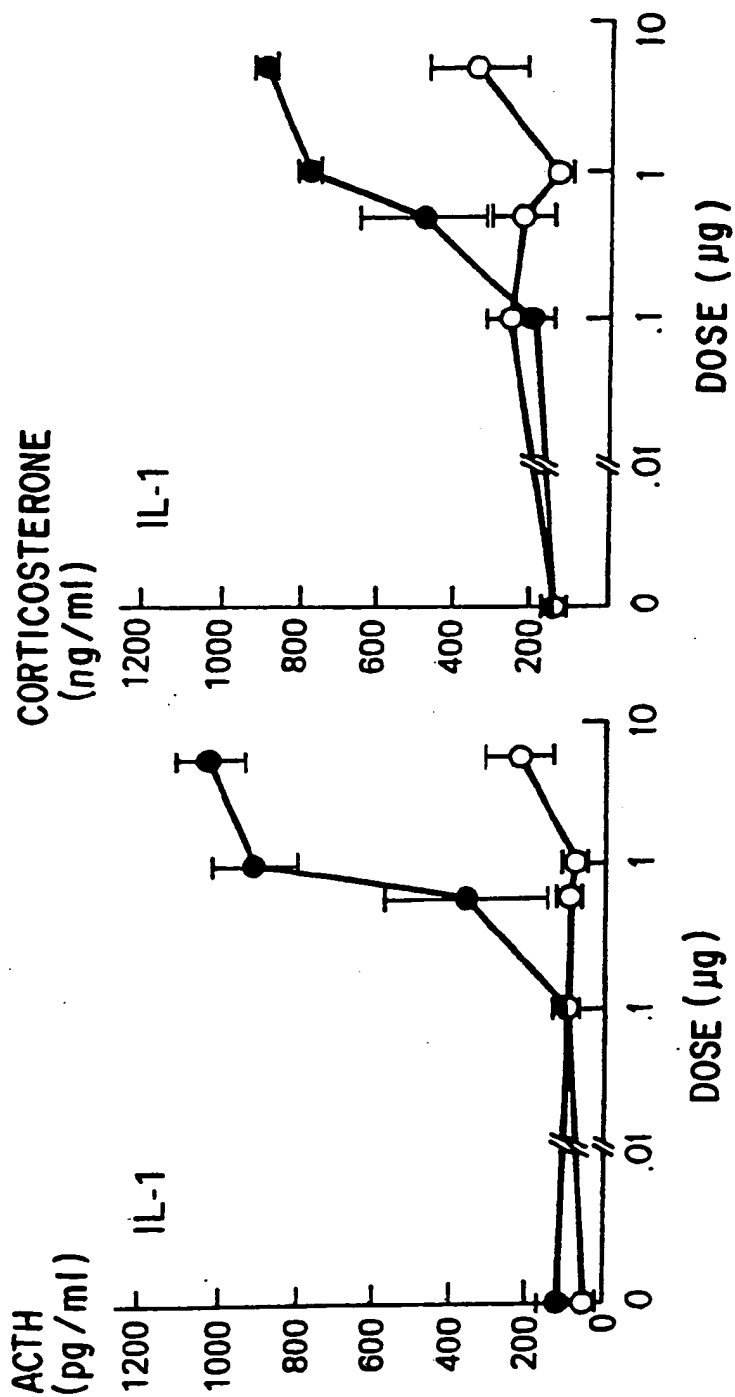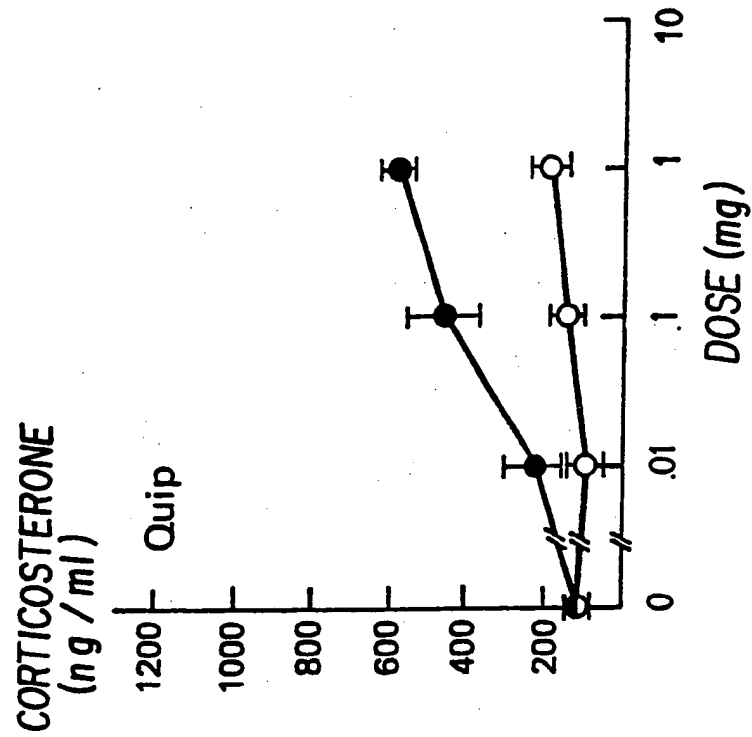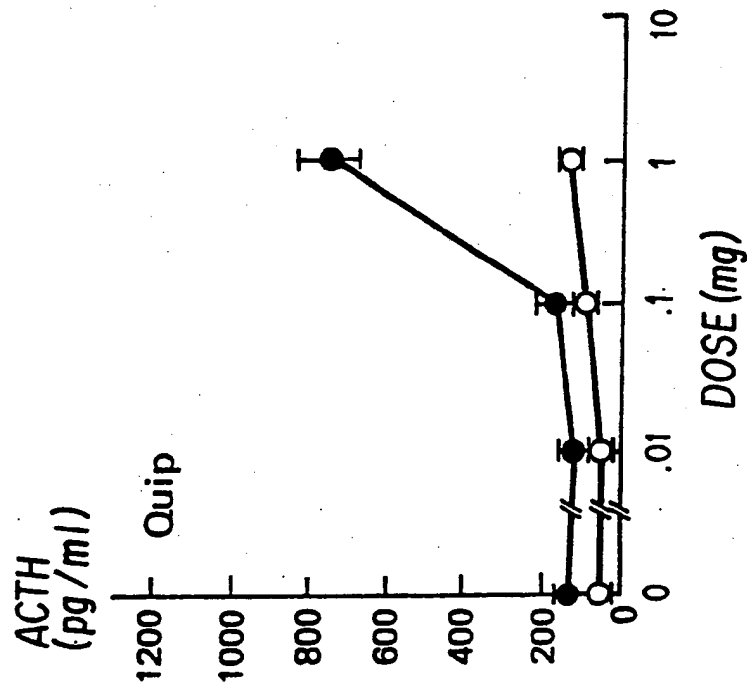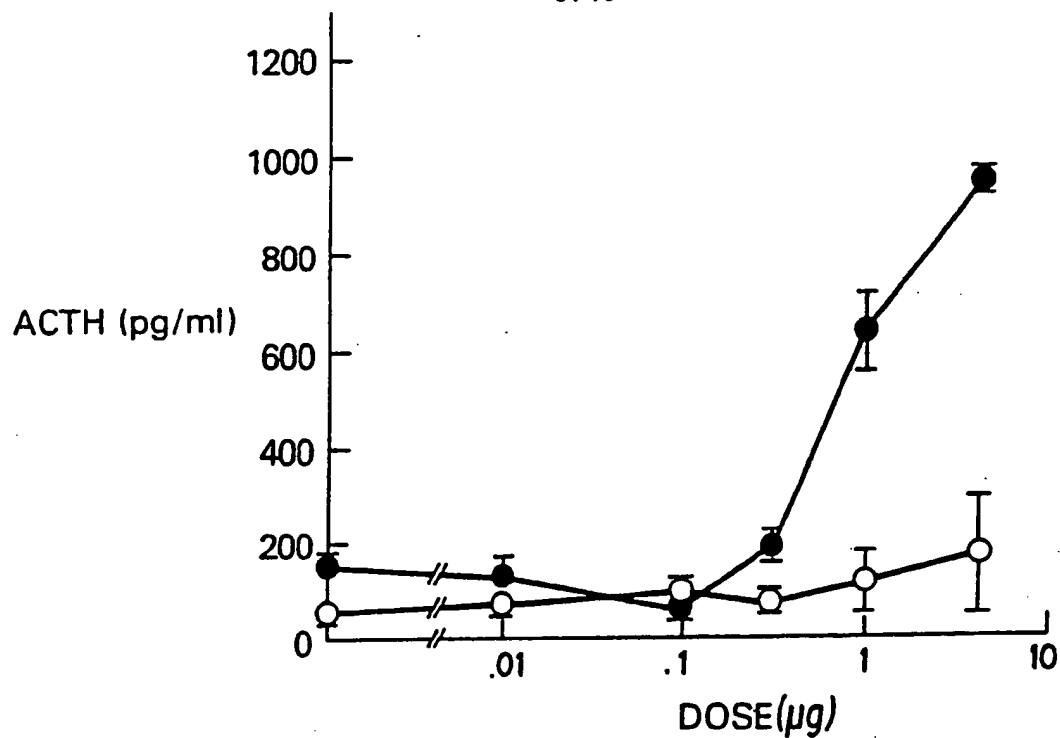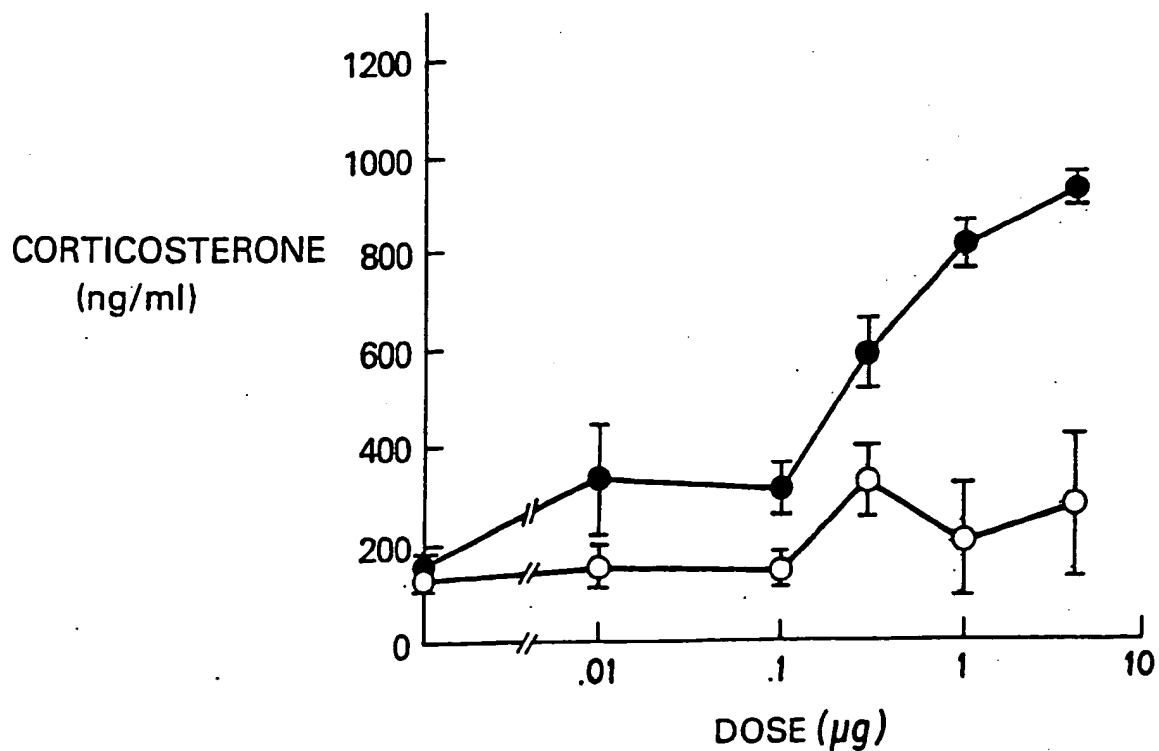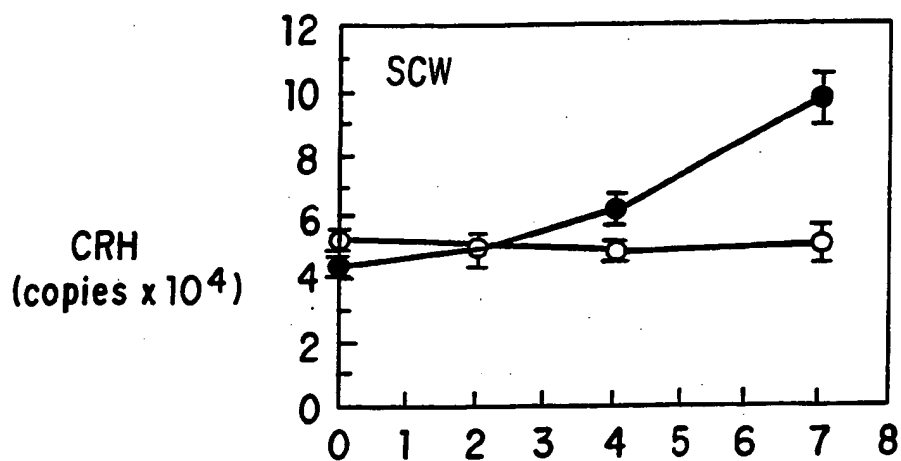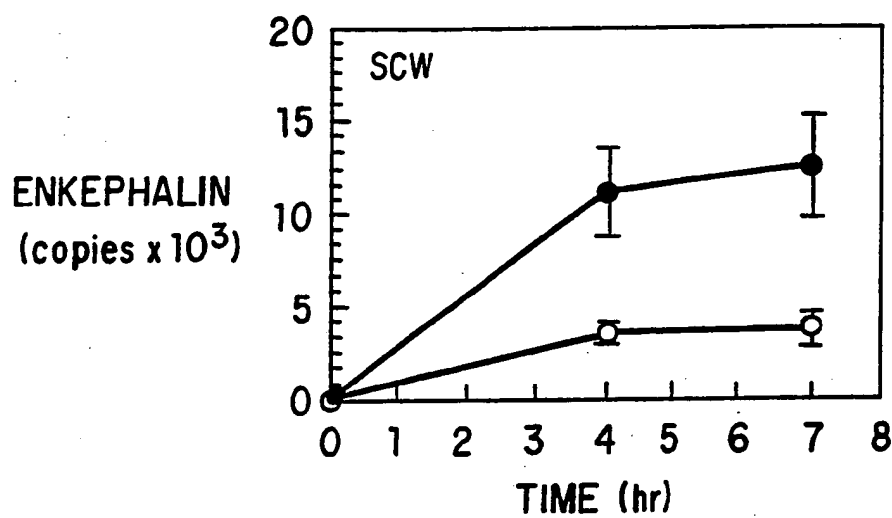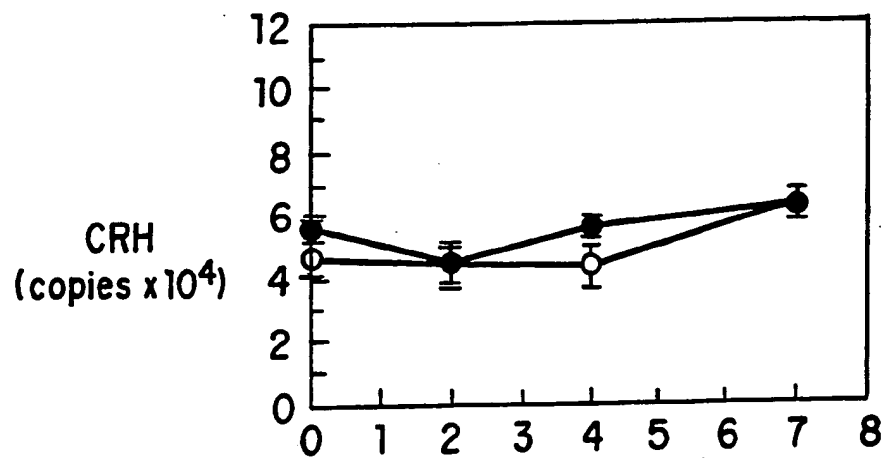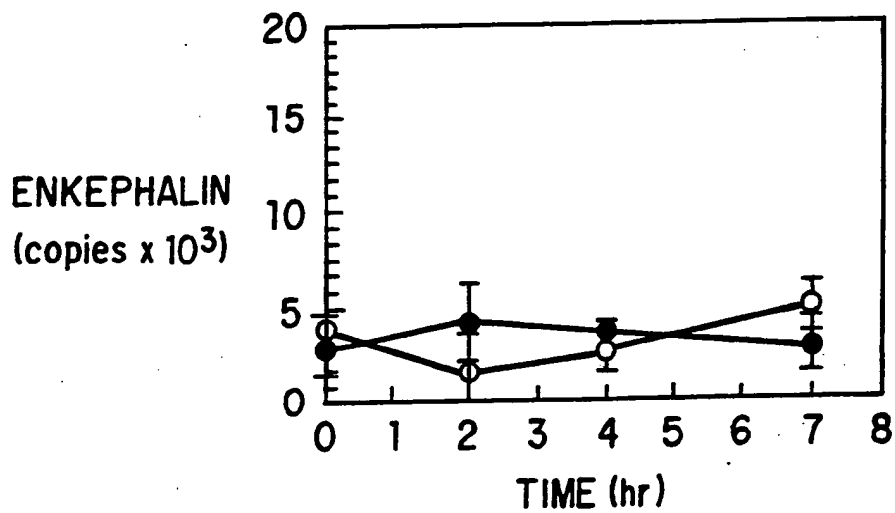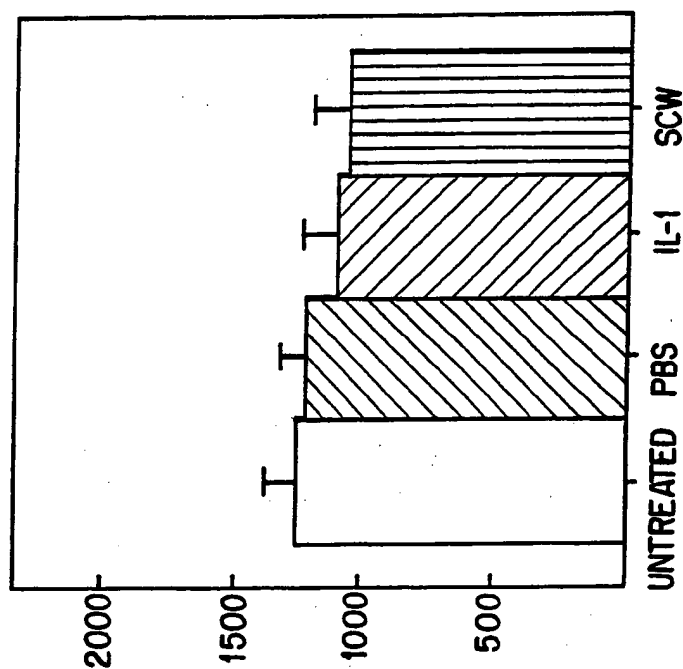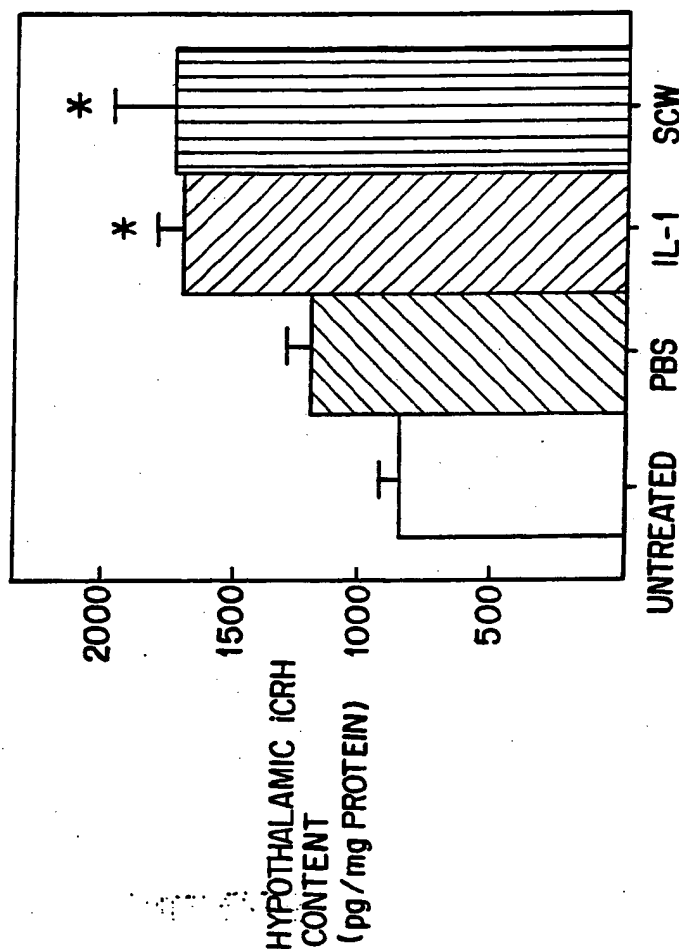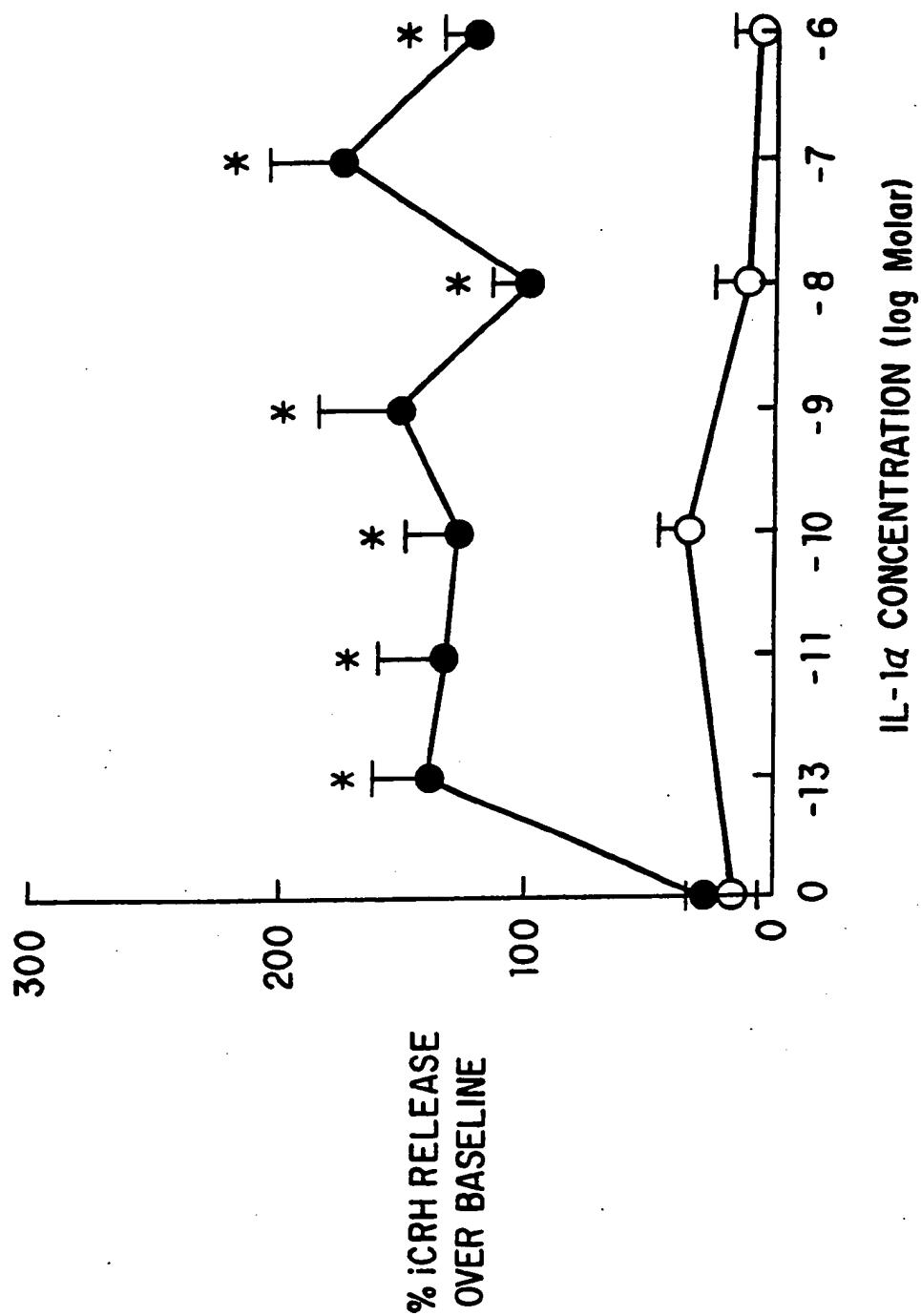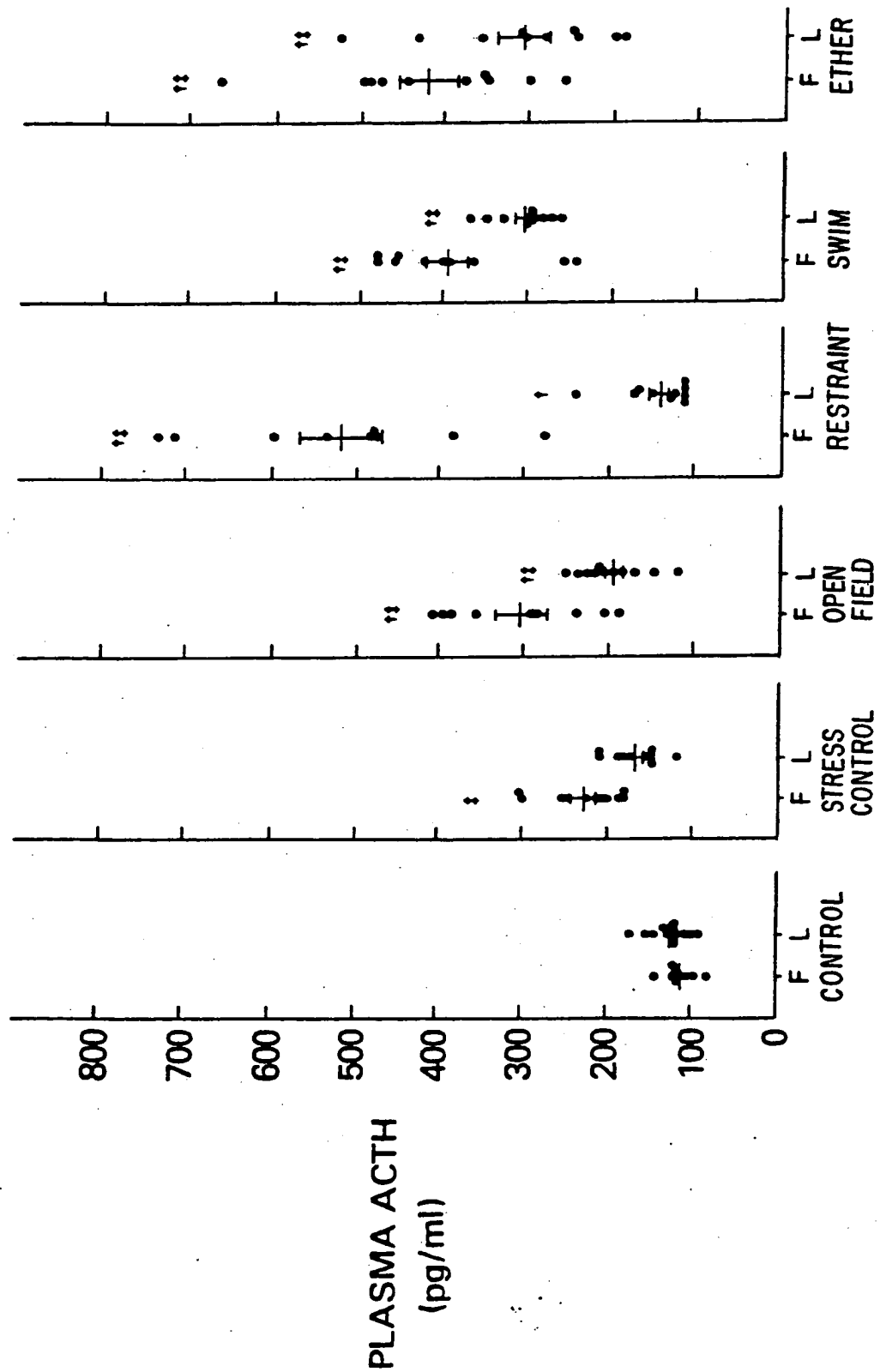
FIG.9A FIG.9B FIG.9C FIG.9D FIG.9E FIG.9F

PLASMA ACTH (pg/ml)

15/15
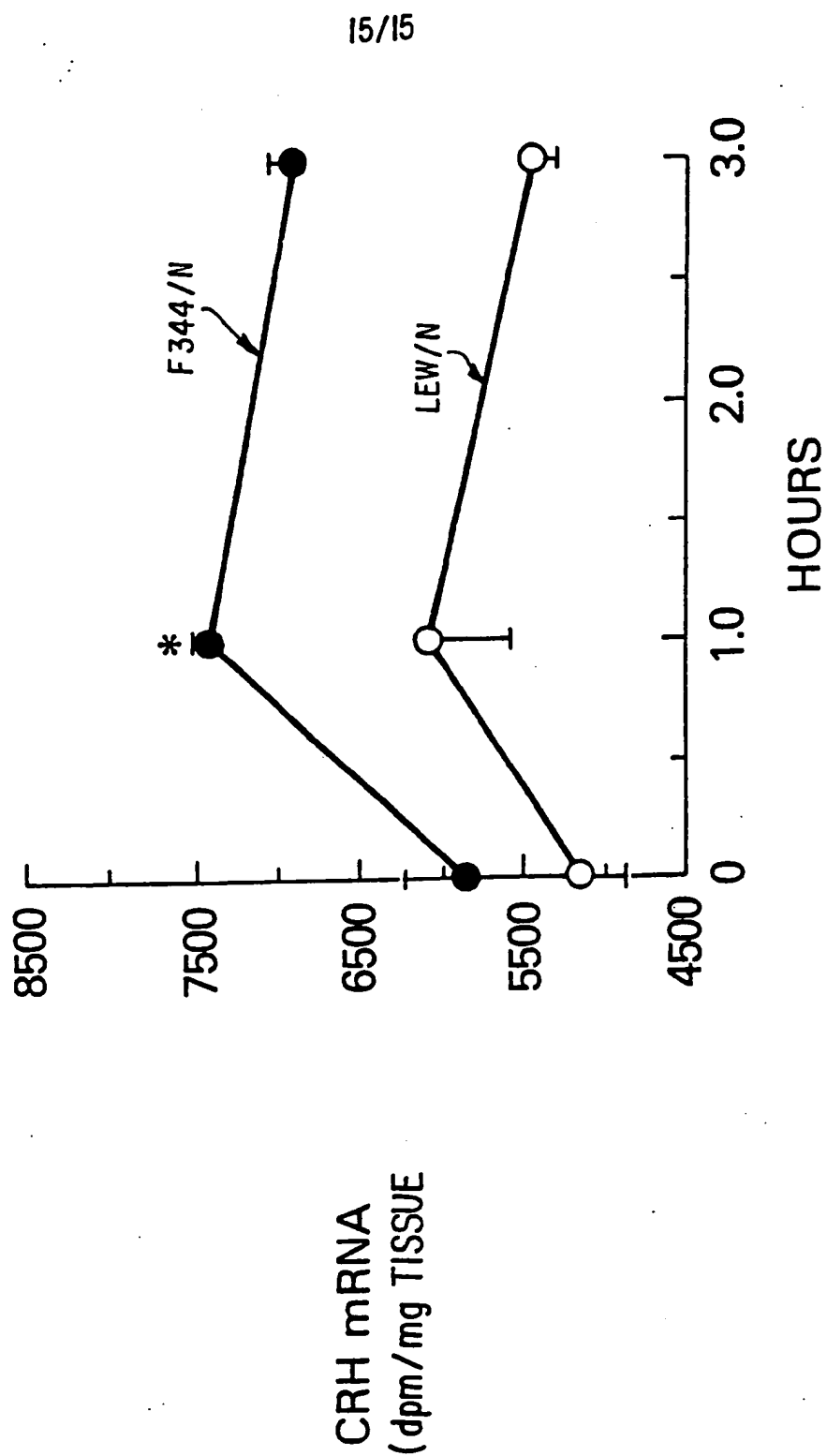
FIG. 10

INTERNATIONAL SEARCH REPORT

International Application . **PCT/US90/05457**

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)
According to International Patent Classification (IPC) or to both National Classification and IPC
IPC (5): G01N 1/00, 33/48; A61K 37/66, 45/05; U.S. CL. 514/889   U.S. CL. 424/2, 9, 85.1, 85.2, 85.4-85.7; 514/805,825,885,886

II. FIELDS SEARCHED

Minimum Documentation Searched [7]	
Classification System	Classification Symbols
U.S.	424/2, 9, 85.1, 85.2, 85.4, 85.5, 85.6, 85.7 514/805, 825, 885, 886, 889

Documentation Searched other than Minimum Documentation   to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [*]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Journal of Immunology, Vol. 129, No. 5, November 1982, David S. Snyder, "Corticosteroids Inhibit Murine Macrophage Ia Expression And Interleukin 1 Production" See Materials ans Methods and Results sections.	1-10,20-28
X	New England Journal of Medicine, Vol. 310, No. 10, 08 March 1984, George P. Chrousos, "The Corticotropin-Releasing Factor Stimulation Test" See Abstract.	1-10,20-28
X	Science, Vol. 230, pages 1035-1037, 29 November 1985, B.M.R.N.J. Woloski, "Corticotropin-Releasing Activity of Monokines" See entire document.	1-10,20-28
X	Science, Vol. 233, pages 652-654, 08 August 1986, Hugo Besedovsky, "Immunoregulatory Feedback Between Interleukin-1 and Glucocorticoid Hormones" See Abstract.	1-10,20-28

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
11 JANUARY 1991	11 FEB 1991
International Searching Authority	Signature of Authorized Officer
ISA/US	Nathan M. Nutter

Form PCT/ISA/210 (second sheet) (Rev.11-87)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V.☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1 ☐ Claim numbers _____ . because they relate to subject matter [12] not required to be searched by this Authority, namely:

2.☐ Claim numbers _____ . because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3.☐ Claim numbers _____ , because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI.☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this international application as follows:

   I. Claims 1-10 and 20-28 drawn to a method and use of a compound for detection of inflammatory diseases.

II. Claims 11-19 drawn to a test kit.

1.☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

     1-10 & 20-28

4.☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (supplemental sheet (2) (Rev. 11-87)

III DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	Science, Vol. 238, pages 519-526, 23 October 1987, Edward W. Bernton, "Release of Multiple Hormones by a Direct Action of Interleukin-1 on Pituitary Cells" See Abstract.	1-10,20-28
X	Journal of Clinical Investigation, Inc. Vol. 82, September 1988, pages 767-774, Aldo E. Calogero, "Multiple Feedback Regulatory Loops upon Rat Hypothalamic Corticotropin-releasing Hormone Secretion" See entire document.	1-10,20-28
X	Journal of Clinical Investigation, Inc. Vol. 82, September 1988, pages 839-846, Aldo E. Calogero, "Catecholamine Effects Upon Rat Hypothalamic Corticotropin-releasing Hormone Secretion In Vitro" See Abstract.	1-10,20-28
Y	J. Endocr. Vol. 151-160, 1982, M.C. Holmes, "Role of serotonin in the control of secretion of corticotrophin releasing factor" See Summary.	1-10,20-28
Y	Journal Of Immunology, Vol. 137, No.1, 01 July 1986, Ester M. Sternberg, "Effect Of Serotonin On Murine Macrophages: Suppression Of Ia Expression By Serotonin And Its Reversal By $5-HT_2$ Serotonergic Receptor Antagonists" See Abstract.	1-10,20-28
Y	J. Exp. Med., Vol. 164, July 1986, pages 327-332, Susan C. Ridge, "Adminstration Of Group A Streptococcal Cell Walls To Rats Induces An Interleukin 2 Deficiency" See Abstract.	1-10,20-28
Y	Endocrinology, Vol. 121, No. 4, 01 June 1987, pages 1580-1582, Akira Uehara, "Interleukin-1 Stimulates Acth Release By An Indirect Action Which Requires Endogenous Corticotropin Releasing Factor" See Abstract.	1-10,20-28
A	Journal Of Imunology, Vol. 138, pages 4360-4365, No. 12, 15 June 1987, Ester M. Sternberg, "Effect Of Serotonin (5-HT) And Other Monoamines On Murine Macrophages: Modulation Of Interferon-γ Induced Phagocytosis" See Abstract.	1-10,20-28
A	Nature, Vol. 328, pages 643-645, 13 August 1987, Stafford L. Lightman, "Changes in hypothalamic preproenkephalin A mRNA following stress and opiate withdrawal" See entire document.	1-10,20-28

III. DOCUMENTS CONSIDERED TO BE RELEVANT   (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Science, Vol. 240, pages 1003-1009, 20 May 1988, John A. Todd, "A Molecular Basis for MHC Class II-Associated Autoimmunity" See entire document.	1-10,20-28

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